Copy for the Elected Office (EO/US) PCT/AU00/00822

PATENT COOPERATION TREATY

10/030411

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

A.P.T. Patent and Trade Mark Attorneys
P.O. Box 222
Mitcham
South Australia 5062
AUSTRALIE

RECEIVED
JUN 27 2002
TECH CENTER 1600/2900

Date of mailing (day/month/year) 04 June 2002 (04.06.02)	
Applicant's or agent's file reference 1727PCT	**IMPORTANT NOTIFICATION**
International application No. PCT/AU00/00822	**International filing date** (day/month/year) 07 July 2000 (07.07.00)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address	State of Nationality	State of Residence
A.P.T. Patent and Trade Mark Attorneys GPO Box 772 Adelaide, S.A. 5000 Australia		
	Telephone No. 08 8410 5040	
	Facsimile No. 08 8410 5042	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☐ the name ☒ the address ☐ the nationality ☐ the residence

Name and Address	State of Nationality	State of Residence
A.P.T. Patent and Trade Mark Attorneys P.O. Box 222 Mitcham South Australia 5062 Australia		
	Telephone No. 08 8272 3244	
	Facsimile No. 08 8272 3255	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

☒ the receiving Office
☐ the International Searching Authority
☐ the International Preliminary Examining Authority
☐ the designated Offices concerned
☒ the elected Offices concerned
☐ other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Evelyne DURAND (Fax 338.87.40)
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994) 004890740

PATENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

A.P.T. Patent and Trade Mark Attorneys
GPO Box 772
Adelaide, S.A. 5000
AUSTRALIE

Date of mailing (day/month/year)
26 February 2001 (26.02.01)

Applicant's or agent's file reference
1727PCT

IMPORTANT NOTIFICATION

International application No.
PCT/AU00/00822

International filing date (day/month/year)
07 July 2000 (07.07.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
MEDVET SCIENCE PTY LTD
Level 3
South Wing
IMVS Building
Frome Road
Adelaide, S.A. 5000
Australia

State of Nationality: AU
State of Residence: AU
Telephone No.: 08 8222 3777
Facsimile No.: 08 8222 3779
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
MEDVET SCIENCE PTY LTD
20 Dalgleish Street
Thebarton, S.A. 5000
Australia

State of Nationality: AU
State of Residence: AU
Telephone No.: 8150 7555
Facsimile No.: 8150 7550
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
F. Baechler
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 26 February 2001 (26.02.01)	
International application No. PCT/AU00/00822	**Applicant's or agent's file reference** 1727PCT
International filing date (day/month/year) 07 July 2000 (07.07.00)	**Priority date** (day/month/year) 07 July 1999 (07.07.99)
Applicant SIMMONS, Paul et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   01 February 2001 (01.02.01)

   [ ] in a notice effecting later election filed with the International Bureau on:

   _____

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer F. Baechler
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

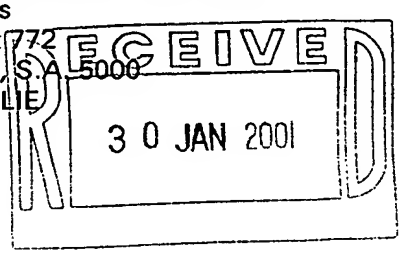
From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
A.P.T. Patent and Trade Mark
Attorneys
GPO Box 772
Adelaide, S.A. 5000
AUSTRALIE



Date of mailing (day/month/year) 18 January 2001 (18.01.01)	
Applicant's or agent's file reference 1727PCT	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/AU00/00822	07 July 2000 (07.07.00)	07 July 1999 (07.07.99)

Applicant
MEDVET SCIENCE PTY LTD et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AG,AU,BZ,DZ,KP,KR,MZ,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   18 January 2001 (18.01.01) under No. WO 01/04268

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

A.P.T. Patent and Trade Mark
Attorneys
GPO Box 772
Adelaide, S.A. 5000
AUSTRALIE

Date of mailing (day/month/year)	
26 February 2001 (26.02.01)	
Applicant's or agent's file reference	**IMPORTANT INFORMATION**
1727PCT	

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/AU00/00822	07 July 2000 (07.07.00)	07 July 1999 (07.07.99)

Applicant
MEDVET SCIENCE PTY LTD et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AG,AL,AM,AT,AZ,BA,BB,BR,BY,BZ,CH,CR,CU,DK,DM,DZ,EE,ES,FI,GB,
   GD,GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MW,
   MX,MZ,PT,SD,SG,SI,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

F. Baechler

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:

A.P.T.
GPO Box 772
Adelaide, S.A. 5000
AUSTRALIE

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
18 August 2000 (18.08.00)	
Applicant's or agent's file reference	**International application No.**
1727PCT	PCT/AU00/00822

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

MEDVET SCIENCE PTY LTD (for all designated States except US)
SIMMONS, Paul et al (for US)

International filing date	:	07 July 2000 (07.07.00)
Priority date(s) claimed	:	07 July 1999 (07.07.99)
Date of receipt of the record copy by the International Bureau	:	24 July 2000 (24.07.00)
List of designated Offices	:	

AP :GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW
EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
National :AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EE, ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA, MD,MG,MK,MN,MW,MX,MZ,NO,NZ,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US, UZ,VN,YU,ZA,ZW

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase
- [ ] confirmation of precautionary designations
- [X] requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

Marie-José Devillard

Telephone No. (41-22) 338.83.38

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

A.P.T. Patent and Trade Mark Attorneys
GPO Box 772
Adelaide, S.A. 5000
AUSTRALIE

Date of mailing (day/month/year)
26 February 2001 (26.02.01)

Applicant's or agent's file reference
1727PCT

IMPORTANT NOTIFICATION

International application No.
PCT/AU00/00822

International filing date (day/month/year)
07 July 2000 (07.07.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
MEDVET SCIENCE PTY LTD
Level 3
South Wing
IMVS Building
Frome Road
Adelaide, S.A. 5000
Australia

State of Nationality: AU
State of Residence: AU
Telephone No.: 08 8222 3777
Facsimile No.: 08 8222 3779
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
MEDVET SCIENCE PTY LTD
20 Dalgleish Street
Thebarton, S.A. 5000
Australia

State of Nationality: AU
State of Residence: AU
Telephone No.: 8150 7555
Facsimile No.: 8150 7550
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
F. Baechler
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

A.P.T. PATENT AND TRADE MARK ATTORNEYS
GPO Box 772
ADELAIDE SA 5001

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *day/month/year*	16 FEB 2001
Applicant's or agent's file reference 1727pct	**IMPORTANT NOTIFICATION**

International Application No.	International Filing Date	Priority Date
PCT/AU00/00822	7 July 2000	7 July 1999

Applicant
MEDVET SCIENCE PTY LTD et al

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translations to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide

Name and mailing address of the IPEA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	PHILIPPA WYRDEMAN Telephone No. (02) 6283 2554

14

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 1727pct	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International Application No. **PCT/AU00/00822**	International Filing Date *(day/month/year)* 7 July 2000	Priority Date *(day/month/year)* 7 July 1999
International Patent Classification (IPC) or national classification and IPC **Int. Cl.** [7] C12N 5/00, 5/08		
Applicant MEDVET SCIENCE PTY LTD et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheet(s).

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

Date of submission of the demand	Date of completion of the report
1 February 2001	13 February 2001
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized Officer **PHILIPPA WYRDEMAN** Telephone No. (02) 6283 2554

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/AU00/00822

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed.

[ ] the description, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages , received on with the letter of

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , received on with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, was on the basis of the sequence listing:

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages

[ ] the claims, Nos.

[ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	**1-51**	**YES**
	Claims	**None**	**NO**
Inventive step (IS)	Claims	**1-51**	**YES**
	Claims	**None**	**NO**
Industrial applicability (IA)	Claims	**1-51**	**YES**
	Claims	**None**	**NO**

2. Citations and explanations (Rule 70.7)

Novelty (N)

All the documents cited in the ISR were category A only. Therefore the claimed invention is not disclosed in any of these patent documents and hence all the claims are novel.

Claims 1-51 meet the criteria set forth in PCT Article 33(2)-(4) for novelty. The prior art published before the priority date does not disclose methods of enriching mesenchymal precursor cells based on at least two markers being either, the presence of markers specific for mesenchymal precursor cells, the absence of markers specific for differentiated mesenchymal precursor cells or the levels of expression of markers specific for differentiated mesenchymal cells..

Therefore the subject matter of these claims is new and meets the requirements of Article 33(2) PCT with regard to the requirement for novelty.

Inventive Step (IS)

The claimed invention is not obvious in the light of any of the cited documents nor disclosed in any obvious combination, nor would the claimed invention be obvious to a person skilled in the art in the light of common general knowledge by itself or in combination with any of these documents.

Industrial Applicability (IA)

The claimed material is considered industrially applicable.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-51	YES
	Claims	None	NO
Inventive step (IS)	Claims	1-51	YES
	Claims	None	NO
Industrial applicability (IA)	Claims	1-51	YES
	Claims	None	NO

2. Citations and explanations (Rule 70.7)

Novelty (N)

All the documents cited in the ISR were category A only. Therefore the claimed invention is not disclosed in any of these patent documents and hence all the claims are novel.

Claims 1-51 meet the criteria set forth in PCT Article 33(2)-(4) for novelty. The prior art published before the priority date does not disclose methods of enriching mesenchymal precursor cells based on at least two markers being either, the presence of markers specific for mesenchymal precursor cells, the absence of markers specific for differentiated mesenchymal precursor cells or the levels of expression of markers specific for differentiated mesenchymal cells..

Therefore the subject matter of these claims is new and meets the requirements of Article 33(2) PCT with regard to the requirement for novelty.

Inventive Step (IS)

The claimed invention is not obvious in the light of any of the cited documents nor disclosed in any obvious combination, nor would the claimed invention be obvious to a person skilled in the art in the light of common general knowledge by itself or in combination with any of these documents.

Industrial Applicability (IA)

The claimed material is considered industrially applicable.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00822

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 005/00, 005/08,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Chem Abs, Medline, WPIDS: enrich?, mesenchym?, purif?,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SIMMONS, P. J. et al (1994) "Isolation, characterisation and functional activity of human marrow stromal progentors in hemopoiesis" *Advances in Bone Marrow Purging and Processing: Fourth International Symposium*, pages 271-280.	
A	GRONTHOS, S. et al (1994) "The STRO-1$^+$ Fraction of Adult Human Bone Marrow Contains the Osteogenic Precursors" *Blood*, vol. 84, No. 12, pages 4164-4173.	

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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MESENCHYMAL PRECURSOR CELL

This invention relates to the enrichment of mesenchymal precursor cells using a combination of cell surface markers, and to a cell population of mesenchymal precursor cells.

Mesenchymal cells are derived from a number of tissues and act as the supportive structure for other cell types. Bone marrow for instance is made of both haematopoietic and mesenchymal derived cells. The mesenchymal cells include endothelial cells that form the sinuses and advetitial reticular cells that have characteristics consistent with adipocytes, fibroblasts and muscle cells.

It is believed that certain mesenchymal precursor cells (MPCs) are responsible for the formation of mesenchymal cells. In the bone MPCs are the formative pluripotent blast cells that are believed to be capable of differentiating into any of the specific types of connective tissues (ie. the tissue of the body that support the specialised elements, particularly adipose, areolar, osseous, cartilaginous, elastic and fibrous connective tissues) depending upon the various environmental influences.

Purification or at least enrichment of MPCs is desirable for a variety of therapeutic reasons. The reasons include regeneration of missing or damaged skeletal tissue, enhancing the implantation of various plastic or metal prosthetic devices through the attachment of the isolated and culturally expanded marrow derived mesenchymal cells onto the porous surfaces of the prosthetic devices, which upon activation and subsequent differentiation of marrow-derived mesenchymal cells produce natural osseous bridges.

Composite grafts of cultured mesenchymal cells might be used to augment the rate of haematopoietic cell reserve during bone marrow transplantation.

A class of defects that may be repaired by cultured marrow-derived mesenchymal cells expanded from the MPCs of the present invention is the class of large skeletal defects in bone caused by injury or produced by the removal of large sections of bone infected with tumour. Under normal circumstances this type of defect does not heal and creates nonunion of the bone. This type of defect may be treated by implanting cultured mesenchymal cells contained in calcium phosphate ceramic vehicles into the defect site.

A second class of defect that may be repaired by cultured marrow-derived mesenchymal cells expanded from the MPCs of the present invention, is the damaged articular cartilage generated by trauma or by diseases such as osteoarthritis and rheumatoid arthritis. Under normal circumstances, damage to articular cartilage does not heal except in very young individuals where the underlying bone is also damaged so that a bloody wound is created. It is projected by the present invention that this type of defect can be treated by implanting cultured marrow derived mesenchymal cells into the defect. The cells will be formatted in carriers which will hold the cells in the defect and present them in a manner (round cell morphology) that they differentiate into chondrocytes.

It is not clearly understood why composite grafts of cultured mesenchymal cells and ceramic induce recruitment of haematopoietic stem cells and other marrow elements, however, the fact that this does occur allows for the use of these grafts in a way to sequester haemopoietic stem cells and generate a haematopoietic stem cell reservoir. The reservoir of haematopoietic stem cells can then be used in clinical applications such as marrow transplantation as an alternative method for harvesting haematopoietic stem cells.

Another potential use for purified cells is as a means of gene therapy, by the introduction of exogenous nucleic acids for the expression of therapeutic substances in the bone marrow - see US 5591625 by Gerson *et al.*

A purified source of MPCs is desirable for a number of reasons. One major reason is that if there is a mixed population, MPCs will respond to signals elicited by other cells to behave in a manner that might not be desired. Thus, for example, a contaminating cell might express a cytokine that evokes differentiation into adipose tissue, whereas one may require the cells for bone formation, in which case the usefulness of the MPCs is somewhat limited. Additionally for a reason similar to that given above, purified progenitor cells tend to be easier to handle and manage than less purified cells.

There have been many attempts at purifying or significantly enriching MPCs, however significant enrichment has until the present invention not been achieved. In contrast to the haemopoietic system, in which stem cells can be physically separated based upon differences in their expression of cell surface markers, the cell surface antigenic phenotype of MPCs remains relatively poorly defined. A further problem of purification of MPCs is a result of the physical association between mesenchymal cells and other cell types.

The bone and bone marrow (BM) tissues contain a phenotypically diverse population of stromal cell lineages that are currently thought to arise from a rare and primitive population of multi-potential mesenchymal precursor cells (MPC) [Owen, 1985; Owen and Friedenstein, 1988]. Bone marrow MPC can be readily measured by their ability to form adherent clonogenic clusters composed of fibroblastic-like cells (CFU-F: colony-forming-unit-fibroblast) in short- term liquid culture [Friedenstein *et al*, 1970; Castro-Malaspina *et al*, 1980]. *In vitro* studies have documented variations in the morphology and proliferative capacity of different BM MPC clones [Friedenstein *et al*, 1970; 1976; Castro-Malaspina *et al*, 1980; Owen *et al*, 1987; Bennett *et al*, 1991; Simmons and Gronthos, 1991]. The heterogeneous nature of the BM MPC population was further demonstrated in studies where culture expanded MPC clones displayed different developmental potentials in the presence of glucocorticoids or when transferred into ectopic sites *in vivo* [Friedenstein *et al*, 1980; Owen *et al*, 1987; Bennett *et al*, 1991]. Collectively, these studies support the concept of a stromal cell hierarchy of cellular differentiation by analogy with the haemopoietic system.

Given the extensive literature regarding the characterisation of haemopoietic stem cells and their progeny there has been little progress towards the identification of the various elements which constitute the bone marrow stromal precursor compartment. This is due in part to the low incidence of MPC in aspirates of marrow (0.05% to 0.001%) [Castro-Malaspina *et al* 1980; Simmons and Torok-Storb, 1991a; 1991b; Falla *et al*, 1993; Waller *et al*, 1995a], and because of the paucity of antibody reagents that allow for the precise identification and isolation of the MPC population. Stromal precursor cells have been partially enriched from bone marrow aspirates through their binding to different lectins such as soya bean agglutinin and wheat germ agglutinin or by using a negative immunoselection process based on their lack of expression of various cell surface antigens restricted to the myeloid, erythroid and lymphoid cell lineages [Simmons and Torok-Storb 1991a; 1991b; Simmons *et al*, 1994; Rickard *et al*, 1996]. However, the inefficiency of these selection strategies has resulted in the presence of contaminating populations of accessory cells and haemopoietic progenitor cells. Moreover, a major difficulty in using techniques such as fluorescense activated cell sorting (FACS) to positively select for pure populations of MPC is that they share many common antigens with HSC including early developmental markers such as the human CD34 antigen and the murine stem cell antigen-1.

Recent advances in the study of human stromal stem cell biology have been attributed to the development of novel monoclonal antibodies (Mabs) which recognise antigens on BM MPC that are correspondingly not reactive with haemopoietic progenitors. We have previously described a monoclonal antibody, STRO-1 which identifies an as yet unidentified 60 kDa cell surface antigen expressed on all assayable MPC in aspirates of adult human BM [Simmons and Torok-Storb, 1991a]. The majority of the STRO-1$^+$ bone marrow mononuclear cells (BMMNC) (approximately 90%) have been identified as late stage glycophorin A$^+$ erythroblasts. The MPC population are restricted to the minor population of STRO-1$^+$ cells which lack glycophorin A [Simmons and Torok-Strob, 1991a]. Importantly, STRO-1 demonstrates no detectable binding to haemopoietic progenitors (CFU-GM, BFU-E, BFU-Meg, CFU-GEMM) nor to their precursors (pre-CFU) [Simmons and Torok- Storb, 1991a; Gronthos and Simmons, unpublished observations].

A systematic examination of the immunophenotype of MPC derived from adult human BM has previously been performed using two-color FACS analysis [Simmons *et al*, 1994]. A number of antigens were shown to be coexpressed with STRO-1 by essentially all BM MPC. These included the endopeptidases CD10 and CD13 and the adhesion molecules Thy-1 (CDw90), VCAM-1 (CD106) and various members of the β1 (CD29) integrin family [Simmons *et al*, 1994]. This is in accord with the data of Terstappen and colleagues regarding the antigenic phenotype of human foetal BM MPC [Waller *et al*, 1995].

SUMMARY OF THE INVENTION

This invention arises from the finding that enrichment of mesenchymal precursor cells is greatly enhanced by the use of two markers specific for mesenchymal cells, that can be used to recognise early cells. To this end it will be appreciated that MPCs are early cells that are substantially at a pre-expansion stage of development and hence are precursors to mesenchymal stem cells in which a significant number of the population have expanded and are therefore incapable of further expansion. Thus, MPCs are cells that have yet to differentiate to fully committed mesenchymal cells. These cells need not however be stem cells in a strict sense, in that they are necessarily able to differentiate into all types of mesenchymal cells. There is a benefit in having an enriched pool of MPCs that are able to differentiate into bone forming cells only, in that these precursor cells have a greater proliferation potential. In particular in accordance with the present invention because the proportions of MPCs in the harvested population is large, the extent to which the population can be expanded is greatly enhanced.

The present invention provides an enrichment several orders of magnitude better than the best method known to the inventors before the present invention. The inventors have shown that an enriched population in which up to 50% of the MPCs can form colonies of ten or more cells can be achieved using the present invention. In contrast, the citations indicate that the best method known up until now has only achieved an enrichment of up to 0.01% cells capable of forming colonies. It is to be noted that as discussed herein the presence of MPCs is based upon their colonigenic capacity, as determined by the presence of colonies of ten or more cells in liquid culture seeded with single cells after having been grown for 14 days.

In a broad form of a first aspect the invention could be said to reside in a method of enriching mesenchymal precursor cells (MPCs) the method including the steps of enriching for cells based on at least two markers, said markers being either the presence of, or expression levels of markers specific for mesenchymal precursor cells on the one hand, or absence of marker or levels of expression specific for differentiated mesenchymal cells on the other hand.

The preferred source of material for enrichment is bone marrow, and thus in a one form the method is limited to the enrichment of bone marrow derived mesenchymal stem cells. It is also likely that the method of this first aspect of the invention might be used to enrich stromal stem cells from other sources such as blood, epidermis and hair follicles. It is proposed that mesenchymal precursor cells isolated from, for example, skin should have the same potential as those cells isolated from bone marrow. An advantage in isolating cells from skin is that the harvesting is far less invasive than the harvesting of a sample of bone marrow.

It is anticipated that a proportion of the population purified will be stem cells, however, it is not yet known how to separate these stem cells from the MPC population. It has been observed however that a subpopulation has a much greater capacity to divide than others, and perhaps this subpopulation represents the stem cells. It is estimated that approximately 10 to 20% of the MPCs isolated by the illustrated method of this invention are stem cells.

It is preferred that a significant proportion of the MPCs are capable of differentiation into at least two committed cell types selected from the group including but not limited to adipose, areolar, osseous, cartilaginous, elastic and fibrous connective.

It has been found that it is possible to purify MPCs by the above method to a degree where these cells are present in a purified population of which 50% of the MPCs can form colonies of ten or more cells. Therefore the method may result in a cell population in which at least 1% of the cells are MPCs that are colony forming, preferably at least 5% of the cells are MPCs that are colony forming, more preferably at least 10% of the cells are MPCs that are colony forming, and most preferably at least 40% of the cells are MPCs that are colony forming.

The nearest known purification is that by Pittenger *et al.* (Science 284; 143-147) where cells had been enriched using a Percoll gradient. These workers were only able to get colony forming units from 0.001 - 0.01% of cells. The present technique therefore results in a very significant enrichment when compared to these attempts.

The present invention is also to be contrasted to the enriched populations described by Caplan *et al.* in US patent 5,837,539 who describes a method for the isolation, purification and culture expansion of mesenchymal stem cells which is said to give compositions having greater than 95% human mesenchymal stem cells. It is to be noted that the figure of 95% relates to populations of expanded mesenchymal stem cells, and is likely to reflect a lower number of colony forming units because the cells are at least partially expanded. Thus, Caplan starts with a population of BM cells comprising about 1 in 1000 MPCs and expands the population and then purifies the at least partially expanded population. In contrast the present invention can result in a population of about 1 in 2 cells that are able to form colonies of at least 10 MSCs.

Preferably the method includes enriching by selecting for the positive expression of at least one marker and more preferably both markers are selected for positive expression. These markers are most conveniently cell surface markers. The markers might be selected from a group of surface markers specific for MPC including but not limited to LFA-3, THY-1, VCAM-1, ICAM-1, PECAM-1, P-selectin, L-selectin, CD49b/CD29, CD49c/CD29, CD49d/CD29, CD29, CD18, CD61, 6-19, thrombomodulin, CD10, CD13, SCF, and the antigen recognised by STRO-1.

Reagents suitable for use in labelling these markers can be found in Table 4.

The marker might be absence of various surface markers indicative of commitment, such as CBFA-1, collagen type II, PPARγ2, glycophorin A.

In one preferred form at least one of the markers is the antigen recognised by STRO-1, and in particular the high level of expression of that antigen.

In another preferred form at least one of the markers is VCAM-1.

In one very specific form the two markers are the antigen recognised by STRO-1 and VCAM-1.

The specificity of the markers used in this process is not absolute. Thus even the most preferred markers occur on cell types other than mesenchymal cells, however their expression on the cell surfaces of other cell types is limited.

It will be understood that recognition of cells carrying the cell surface markers that form the basis of the separation can be effected by a number of different methods, however, all of these methods rely upon binding a binding agent to the marker concerned followed by a separation of those that exhibit binding, being either high level binding, or low level binding or no binding. The most convenient binding agents are antibodies or antibody based molecules, preferably being monoclonal antibodies or based on monoclonal antibodies because of the specificity of these latter agents. Antibodies can be used for both steps, however other agents might also be used, thus ligands for these markers may also be employed to enrich for cells carrying them, or lacking them.

The antibodies may be attached to a solid support to allow for a crude separation. The separation techniques should maximise the retention of viability of the fraction to be collected. Various techniques of different efficacy may be employed to obtain relatively crude separations. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill. Procedures for separation may include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity chromatography and "panning" with antibody attached to a solid matrix. Techniques providing accurate separation include but are not limited to FACS.

The method might include the step of making a first partially enriched pool of cells by enriching for the expression of a first of the markers, and then the step of enriching for expression of the second of the markers from the partially enriched pool of cells.

It is preferred that the method comprises a first step being a solid phase sorting step, based on recognition of one or more of the markers. The solid phase sorting step of the illustrated embodiment utilises MACS recognising high level expression of STRO-1. This then gives an enriched pool with greater numbers of cells than if a high accuracy sort was used as a first step. If for example FACS is used first, many of the MPCs are rejected because of their association with other cells. A second sorting step can then follow using an accurate separation method. This second sorting step might involve the use of two or more markers. Thus in the illustrated embodiment two colour FACS is used to recognise high level expression of the antigen recognised by STRO-1 as wells as the expression of VCAM-1. The windows used for sorting in the second step can be more advantageously adjusted because the starting population is already partially enriched.

It will be understood that the invention is not limited to the enrichment of cells by their expression of only two markers and it may be preferred to enrich based on the expression of three or more markers.

The method might also include the harvesting of a source of the stem cells before the first enrichment step, which in the most preferred source comprises the step of harvesting bone marrow cells, using known techniques.

The preferred source of such cells is human, however, it is expected that the invention is also applicable to animals, and these might include domestic animals or animals that might be used for sport.

In a broad form of a second aspect the invention could be said to reside in an enriched population of mesenchymal precursor cells as purified by a method according to the first aspect of the invention.

It has been found that it is possible to purify MPCs to a degree where the purified population contains 50% of these cells that are capable of forming colonies of 10 or more cells.

In a broad form of a third aspect the invention could also be said to reside in a cell population in which at least 1% of the cells are MPCs that are colony forming, preferably at least 5% of the cells are MPCs that are colony forming, more preferably at

least 10% of the cells are MPCs that are colony forming, and most preferably at least 40% of the cells are MPCs that are colony forming.

The cells of the enriched population preferably carry at least two markers selected from a group of surface markers specific for mesenchymal precursor cells including LFA-3, THY-1, antigen identified by STRO-1, VCAM-1, ICAM-1, PECAM-1, P-selectin, L-selectin, CD49b/CD29, CD49c/CD29, CD49d/CD29, CD29, CD18, CD61, 6-19, thrombomodulin, CD10, CD13 and SCF. Most preferably the cells carry the antigen identified by STRO-1 and VCAM-1.

It will also be understood that in a fourth aspect the invention encompasses a composition including the purified MPCs or a composition made from the purified MPCs.

The purified population of the second or third aspects of the invention, or the composition of the fourth aspect of the invention might be used in the formation and repair of bones, and as such a combination of MPCs as well as a suitable support may be introduced into a site requiring bone formation. Thus, for example, skeletal defects caused by bone injury or the removal of sections of bone infected with tumour may be repaired by implanting cultured MSCs contained in calcium phosphate ceramic vehicles into the defect site. For appropriate methods and techniques see Caplan *et al.* in US patent 5,226,914 and US patent 5,837,539, both of which use cruder preparations of stem cells.

In addition, the enriched population or composition may be used to assist in anchoring prosthetic devices. Thus, the surface of a prosthetic device such as those used in hip, knee and shoulder replacement, may be coated with the enriched MPCs prior to implantation. The MPCs may then differentiate into osteogenic cells to thereby speed up the process of bony ingrowth and incorporation of the prosthetic device (see Caplan *et al.* in US patent 5,226,914 and US patent 5,837,539).

The enriched population or composition might also be used in gene therapy so that, for example, an enriched population may have exogenous nucleic acid transformed into it and then such a population may be introduced into the body of the patient to treat a disease or condition. Alternatively it might be used for the release of therapeutics. For appropriate techniques we refer to US patent 5591625 by Gerson *et al.* which uses cruder preparations of stem cells.

Alternatively the enriched population or composition may be used to augment bone marrow transplantation, wherein the composition containing purified MSCs can be injected into a patient undergoing marrow transplantation prior to the introduction of the whole marrow. In this way the rate of haemopoiesis may be increased, particularly following radiation or chemotherapy. The composition might also encompass a mixture of MPCs and haemopoietic cells which may be useful in radiotherapy or chemotherapy.

FIGURE LEGENDS

Figure 1 The frequency histogram represents the immunofluorescence analysis by FACS of BMMNC isolated by MACS on the basis of STRO-1 (FITC) expression: STRO-1^{dull} cell fraction (A); STRO-$1^{intermediate}$ cell fraction (B); STRO-1^{bright} cell fraction (C); The histogram is based on 10^4 events collected as list mode data.

Figure 2 Dual-colour flow cytometric analysis of VCAM-1 (PE) expression by STRO-1^+ (FITC) BMMNC isolated by MACS. The dot plot histogram represents 5 x 10^4 events collected as listmode data. STRO-1^{bright}/VCAM-1^+ cells were sorted by FACS (rectangle), which represented approximately 0.1% of the total BMMNC population (A). The incidence of clonogenic cells (B) colonies (>50 cells) and (C) colonies + clusters (>10<50 cells) based on STRO-1^{bright}/VCAM-1^+ expression. The frequency of clonogenic cells was determined by limiting dilution analysis (24 replicates per cell concentration) employing Poisson distribution analysis.

Figure 3 Characterization of BM MPC. (A) Light microscopic examination of the freshly sorted cells revealed a homogenous population of large cells with heterochromatic nuclei and prominent mucleoli, a granular cytoplasm and numerous blel-like projetions of the cell membrane (magnified 40X). (B) Transmission electron micrograph of STRO-1^{bright}/VCAM-1^+ sorted cells isolated directly from

BM (magnified 1000X). (C) Immunohistological staining of cytospin preparations of the sorted STRO-1^{bright}/VCAM-1^{+} BMMNC showing intense staining of most cells with anti-collagen type I antibody, (magnified 40X). (D) Light microscopic view of a purified STRO-1^{bright}/VCAM-1^{+}, allowed to adhere to fibronectin-coated culture adopts a stellate, fibroblastoid morphology.

Figure 4 Characterization of BM MPC. Dual-colour flow cytometric analysis of Ki67 (FITC) expression by STRO- 1^{+} (PE) BMMNC isolated by MACS. The dot plot histogram represents 5 x 10^4 events collected as listmode data (B). Telomerase activity in sorted cells populations was examined using a modified TRAP assay (C). TRAP products derived from CHAPS extracts of non-denatured (-) and denatured (+) total bone marrow (lanes 1 and 2), Total STRO-1 [MACS-selected] (lanes 2 and 3). STRO-1^{bright}/VCAM-1^{+} cells sorted fraction (lanes 4 and 5), cultured. STRO-1^{bright}/VCAM-1^{+} cells (lanes 6 and 7) and CD34^{+}-sorted cells TRAP products were resolved on a 12% polyacrylamide gel, stained with SYBR green fluorescent dye, and visualised using a fluorescence scanning system.

Figure 5 A total of 44 CFU-F colonies derived from two BM samples were analysed for their cumulative production of cells. A marked variation in prolifertive capacity between individual MPC is evident. The majority of clones (36/44; 82%) exhibited only modertate growth potential which did not persist beyond 12 population doublings. 8/44 clones (18%) demonstrated continued growth extending beyond 17 doublings. All clones were switched to adipogenic growth conditions, and under these conditions, 14/44 clones (32%) exhibited adipogenesis.

Figure 6 RT-PCR analysis of gene expression in STRO-1^{bright}/VCAM-1^{+} purified stromal precursor cells (MPC) isolated directly from marrow aspirates, non-induced

primary stromal cultures derived from MPC (CFU-F), and CFU- F cultured under osteogenic- (BONE), chondrogenic- (CART) and adipogenic- (FAT) inductive growth conditions. Various markers of: BONE [transcription factor CBFA1; collagen type I (COLL-I); bonesialoprotein (BSP); osteopontin (OP); osteonectin (ON); osteocalcin (OCN), parathyroid hormone receptor (PTHR)]; FAT [lipoprotein lipase (LPL), transcription factor PPARγ2, leptin, human adipocyte lipid binding protein (H-ALBP)]; CARTILAGE [collagen type II (COIL-II), collagen type X (COLL-X), Aggrecam (AGGN)]. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining.

Figure 7 In *vitro* developmental potential of MPC. Primary cultures of derived from STRO-1^{bright}/VCAM-1+ BMMNC were cultured for 2 weeks then induced under either osteogenic, adipocytic or chondrocytic conditions for 3- 5 weeks. A von Kossa positive mineralised matrix formed throughout the cultures within 4 weeks of bone induction (200X) (A). The presence of clusters of lipid containing adipocytes were also detected by oil red-O staining (200X) (B). Cultures were counter stained with haematoxylin.

Figure 8 New bone formation *in vivo*. Immunoselected STRO-1^{bright}/VCAM-1^{+} BMMNC clones, expanded *in vitro*, were implanted subcutaneously into SCID mice using porous ceramic cubes. Implants were harvested 8 weeks post transplant. New bone formation (solid arrow) was observed for a proportion of clones within the cavities of the ceramic cubes (open arrow) together with surrounding fibrous and hematopoietic tissue (40X) (A). The sections were counter stained with haematoxylin and eosin. A magnified view of new bone formation is shown depicting an osteocyte (arrow) (200X) (B).

Figure 9 Dual parameter flow cytometric analysis of STRO-1$^+$ human bone marrow mononuclear cells isolated by MACS. A distinct subpopulation of STRO-1^{bri} cells are identified by VCAM-1, THY-1 (CD90), MUC-18 (CD-146) and STRO-2.

To properly investigate the biology of BM MPC, studies were designed to isolate MPC from a heterogeneous population of unfractionated BM cells. This was achieved by using a combination of positive immunoselection procedures based on the unique specificity of the STRO-1 mab, in order to maximise the recovery and purity of the MPC population. Following the isolation of homogeneous populations of MPC we then explored their pattern of gene expression for various bone-, fat- and cartilage-related markers to determine the degree of commitment towards different stromal cell lineages *in vivo*. Finally we have investigated the developmental potential of purified populations of BM MPC *in vitro* under defined conditions [Gronthos *et al*, 1994] and *in vivo* by ectopic implantation into immunodeficient mice [Haynesworth *et al*, 1992].

We and others have had success in isolating MPC based on their expression of the STRO-1 antigen either by FACS or by using immunomagnetic particles, such as Dynabeads [Tamayo *et al*, 1994] or by magnetic-activated cell sorting (MACS) [Gronthos *et al*, 1995 and 1998]. The latter was used initially to provide a reproducible technique for isolating BM derived MPC with the capacity to process high cell numbers. The mab STRO-1 proved to be an ideal reagent for isolating MPC from adult BM because of its lack of reactivity to haemopoietic progenitors [Simmons and Torok-Storb, 1991a] yielding a clean separation between MPC and haemopoietic progenitors in adult BM. Moreover, the antigen identified by STRO-1 was found in the present study to be expressed at particularly high copy number by MPC, which may in part account for the high efficiency and recovery of BM CFU-F observed. These studies identified the minor STRO-1^{bright} subset of the total STRO-1$^+$ BMMNC fraction to contain the CFU-F population. However the resulting post MACS STRO-1^{bright} cell population was only partially enriched for MPC.

We have previously demonstrated that the cell surface antigen, VCAM-1 is universally expressed on BM MPC and their progeny [Simmons *et al*, 1992, 1994]. This is in contrast to other markers expressed by BM MPC such as THY- 1, CD10, CD13, and thrombomodulin, [Simmons *et al*, 1994] which are also known to react with either

haemopoietic cells and or platelets [Baum *et al*, 1992; Conway and Nowakowski, 1993; Ship and Look, 1993]. The VCAM-1 molecule is a transmembrane glycoprotein with a molecular weight of between 95 and 110 kDa present on the membranes of stromal cells and endothelial cells [Osborn *et al*, 1989; Simmons *et al*, 1992]. The immunoglobulin super family member is one ligand for the integrin receptor $\alpha4\beta1$ (VLA-4) present on haemopoietic stem cells, and is involved in the recruitment of lymphocytes and monocytes expressing $\alpha4\beta1$ to sites of infection and inflammation [Elices *et al*, 1990; Simmons *et al*, 1992]. Significantly, VCAM-1 only reacted with a minor proportion of BMMNC effectively subletting the total STRO-1$^+$ population, reacting preferentially with the STRO-1^{bright} cell fraction. The BM MPC population was subsequently shown to reside exclusively in the STRO-1^{bright}/VCAM-1$^+$ fraction of human adult BM.

The absolute frequency of MPC in bone marrow was determined by limiting dilution experiments using Poisson distribution statistics. Other studies using this statistical analysis have shown that murine BM osteoprogenitor cells with the potential to form mineralized bone nodules *in vitro*, occurred at a frequency of 1 per 1000 BM cells plated, based on the phenotype 5-fluoracil resistant, haemopoietic lineage marker negative [Van Vlasselaer, 1994]. These osteoprogenitors represented approximately 20% of the total MPC population in normal murine BM [Falla *et al*, 1993; Van Vlasselaer, 1994]. Similar analyses of fetal human BMMNC demonstrated the frequency of MPC at 1 per 1,000 to 1 per 100,000 cells plated, at 14 weeks and 24 weeks gestation, respectively, based on the immunophenotype CD34$^+$/CD38$^-$/HLA-DR$^-$ [Waller *et al*, 1995a]. Furthermore, additional subletting of fetal BM using the haemopoietic marker CD50, distinguished HSC from the MPC population, but found no significant difference in the incidence of clonogenic stromal cells sorted on the basis of the phenotype CD34$^+$/CD38$^-$/HLA-DR$^-$ /CD50$^-$ [Waller *et al*, 1995b]. However, no stromal progenitors were observed when single cells of human adult BM samples were sorted based on the CD34$^+$/CD38$^-$/HLA-DR$^-$ phenotype [Waller *et al*, 1995a]. This may be due to the inefficiency of a predominantly negative selection criteria used to isolate fetal BM MPC and may also reflect the use of the CD34 antigen which demonstrates low level expression on adult BM MPC [Simmons and Torok-Storb, 1991b].

In the illustrated embodiment, the incidence of clonogenic cells (clusters 10<50 cells + colonies 50) from adult human BM was determined to be 1 per 2 STRO-1^{bright}/VCAM-1$^+$ cells plated in SDM containing PDGF and EGF. Using

serum-deprived medium significantly enhances the incidence of clonogenic growth over that of serum replete cultures, particularly at low plating densities [Gronthos and Simmons, 1995]. It must also be stated that a proportion of the wells which were scored as 'negative' contained cell clusters of less than 10 cells. Therefore, by further refining the CFU-F culture assay, it may be possible to stimulate the growth of MPC in order to increase the overall purity of the MPC population based on the composite STRO-1^{bright}/VCAM-1^{+} phenotype. Nevertheless, the combined purification technique of the illustrated embodiment effectively achieved a several thousand fold increase in the incidence of BM MPC when compared to unfractionated BMMNC.

The cells contained within the STRO-1^{bright}/VCAM-1^{+} BM fraction were found to be a homogeneous population of large cells with extensive cytoplasmic processes existing *in vivo* in a non-cycling state. Other studies have found that MPC residing in the BM are almost entirely non-cycling as shown by ^{3}H thymidine labelling in rodents and by means of the *in vitro* thymidine suicide technique in humans [Castro-Malaspina *et al*, 1980; Castro-Malaspina *et al*, 1981]. This data coincides with the observations that primitive multi-potential stem cells, identified in the other cell systems such as HSC are by definition quiescent cells [Andrews *et al*, 1986; Szilvassy *et al*, 1989; Li and Johnson, 1992]. Given the reported developmental potential of cultured BM MPC *in vitro* and *in vivo* the question arises as to whether these cells are truly representative of an early uncommitted phenotype with multi-potential or whether all or a proportion of the CFU-F are already committed towards a particular stromal cell lineage.

Analysis of the gene expression pattern of purified adult BM MPC in the illustrated embodiment has revealed that many of the genes expressed by CFU-F *in vivo* demonstrate a broad stromal tissue distribution related to osteoblasts, adipocytes and chondrocytes. It is very common to find in the literature that many markers for example osteonectin, osteopontin, and alkaline phosphatase in the bone cell lineage are described as being specific to bone cells, when in fact these markers have a wider tissue distribution. Therefore, it is not surprising to find that MPC identified by STRO-1 share common markers with differentiated stromal cell types. Importantly, specific markers of commitment such as CBFA-1, collagen type II, PPARγ2, [reviewed in Rodan and Harada, 1997] to bone, cartilage and fat respectively were not expressed by the STRO-1^{bright}/VCAM-1^{+} population in fresh BM aspirates. In addition, immunohistochemical examination of STRO-1^{bright}/VCAM-1^{+} sorted BMMNC failed to show any reactivity to the smooth muscle marker α-smooth muscle actin or with the endothelial marker, FVIII. Therefore the MPC residing in the BM seem to exist in an

uncommitted state, and may have the potential under different conditions to develop into a few or all of the stromal elements recognised in the bone marrow microenvironment.

In the present study, cultures of purified STRO-1^{bright}/VCAM-1^{+} human BM CFU-F typically developed a von Kossa positive mineral by twenty one days under osteogenic conditions (ASC-2P, PO_{4i}, DEX). The presence of mineral deposits was demonstrated in all CFU-F clones examined, where 40% of the clones also displayed the capacity to differentiate into adipocytic cell clusters. Moreover, individual CFU-F clones were also found to contain a small proportion of fibroblastic-like cells not associated with either mineralization or lipid accumulation. These fibroblast-like cells may represent as yet undefined stromal populations such as reticular cells, smooth muscle cells, bone lining cells, osteocytes and committed stromal progenitors.

The developmental potential of selected CFU-F clones was further examined *in vivo*. The porous hydroxyapatite coated ceramic cubes reproducibly supported the development of human osteogenic tissue in SCID mouse. This is in agreement with the findings in previous *in vivo* studies using unfractionated rodent and human BM mesenchymal cell cultures [Haynesworth *et al*, 1992a; Krebsbach *et al*, 1997; Kusnetsov *et al*, 1997]. In the present study, pretreating the HA ceramic cubes with purified fibronectin was critical to maximise the number of cells retained in the cubes after loading prior to transplantation (data not shown). Pre-treatment of HA cubes with fibronectin and laminin has been reported to increase cell retention and spreading on the ceramic surface of the cubes [Dennis *et al*, 1992; Dennis and Caplan; 1993]. Fibronectin and laminin coated cubes were found to augment bone formation from cultured rat BM mesenchymal cells at earlier time points in comparison to untreated cubes [Dennis *et al*, 1992; Dennis and Caplan, 1993].

The present study failed to detect cartilage formation in any of the transplantation models used, in contrast to other studies which demonstrated cartilage formation in diffusion chambers transplanted with rodent bone marrow or mesenchymal cells derived from the marrow of young children. To date, there have been no reports describing the reproducible induction of cartilage formation using adult human bone marrow stromal cells *in vivo* or *in vitro*. In the present study, the expression of the hypertrophic chondrocyte marker collagen type X, by purified adult human BM MPC, is somewhat puzzling, given the presumed specificity of this molecule. Since the

physiological role of collagen type X is unknown, its significance in bone marrow remains to be determined.

The present work is in accord with previous studies showing that the formation of new bone in implants of HA cubes is attributed to differentiation of human mesenchymal cells into functional osteoblasts [Kusnetsov *et al*, 1997] and did not result from the recruitment of osteoprogenitors from the surrounding host (mouse) tissue. Furthermore, other cell types present such as muscle, adipocytes and vascular endothelial cells showed no hybridization with the alu probe and are therefore presumed to be host in origin. These findings demonstrate that a proportion of BM MPC within the STRO-1^{bright}/VCAM-1^{+} BM subfraction, demonstrate the capacity to develop into multiple stromal cell types including osteoblasts, adipocytes and fibroblast-like cells.

Further subletting of the STRO-1^{bright}/VCAM-1^{+} BM fraction using three- and four-colour FACS analysis may eventually provide a means to discriminate between subpopulations contained within the MPC pool which exhibit different developmental potentials. The purification of MPC clones with different potential may then be used to generate multipotent, bi-potent and uni-potent cell lines which could greatly facilitate the design of experimental approaches to study the molecular mechanisms regulating the commitment of early precursors into different stromal cell lineages.

One area of potential benefit that will occur from a greater understanding of the proliferation and differentiation of MPC, is the ability to manipulate and expand mesenchymal cell populations *in vitro* for subsequent reimplantation *in vivo*. The use of animal models has demonstrated the efficacy of utilising *ex vivo* expanded BM mesenchymal cells to facilitate bone regeneration and tendon repair *in vivo* [Bruder *et al*, 1998b; 1998c; Young *et al*, 1998]. Several studies have also described how cultured marrow stromal cells from a variety of species are readily infected using either amphotropic retroviruses or adenoviruses [Harigaya and Handa, 1985; Rothstein *et al*, 1985; Singer *et al*. 1987; Cicutinni *et al*, 1992; Roecklein and Torok-Storb, 1995]. In addition, some studies have demonstrated the persistence of transplanted transduced cells over several months in animal models [Li *et al*, 1995; Anklesaria *et al*, 1996; Onyia *et al*, 1998 Reiw *et al*,1998]. Therefore the ability to harvest purified human MPC from aspirates of BM and to expand these cells *ex vivo* makes them ideal candidates as possible vehicles for gene transfer, in order to treat a variety of diseases and genetic disorders.

MATERIALS AND METHODS

Subjects

Aspirates of human BM samples were obtained from the iliac crest and the sternum of normal adult volunteers with their informed consent, according to procedures approved by the ethics committee at the Royal Adelaide Hospital, South Australia. Bone marrow mononuclear cells (BMMNC) were obtained by centrifugation over Ficoll 1.077 g/ml (Lymphoprep, Nycomed, Oslo, Norway) at 400g for 30 minutes (min) and then washed and resuspended with Hank's buffered saline solution containing 1% bovine serum albumin and 10mM HEPES, pH 7.35 (HBSS).

Isolation of STRO-1+ Cells by Magnetic-Activated Cell Sorting (MACS)

This procedure is a modification of that described elsewhere [Gronthos *et al*, 1998]. Approximately 1 x 10^8 BMMNC were incubated with STRO-1 supernatant for 60 min on ice. The cells were then washed in HBSS and resuspended in 1 ml of HBSS containing a 1/50 dilution of biotinylated goat anti-mouse IgM (μ-chain specific; Southern Biotechnology Associates, Birmingham, AL) for 45 min on ice. Following this, the cells were washed twice in MACS buffer (single strength Ca^{2+} and Mn^{2+} free PBS supplemented with 1% BSA, 5mM EDTA and 0.01% sodium azide) and resuspended in 900μl of MACS buffer to which 100μl of streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, F.R.G.) was added. The cells were further incubated for 15 min on ice after which streptavidin-fluorescein isothiosyanate (FITC) conjugate (1/50; Caltag Laboratories, San Francisco, CA) was added directly to the suspension for an additional 5 min. The cells were separated on a Mini MACS magnetic column (column capacity 10^7 cells, Miltenyi Biotec) according to the manufacturers specifications.

Purification of the CFU-F population by fluorescence activated cell sorting (FACS)

Dual colour-FACS analysis of the STRO-1^{bright} population was achieved by incubating the MACS isolated STRO-1 population with saturating levels of the Mab 6G10 (mouse IgG1 anti-human CD106: vascular endothelial adhesion molecule-1, VCAM-1; kindly donated by Dr. B. Masinovski FCOS Corp., Seattle WA) for 30 min on ice. After washing with HBSS the cells were incubated with a second label goat anti-mouse IgG (γ-chain specific) phycoerythrin (PE) conjugate antibody (1/50; Southern Biotechnology Associates, Birmingham, AL) and a streptavidin-FITC conjugate (1/50; CALTAG Laboratories, San Francisco, CA) for 20 min on ice. The cells were then washed in HBSS prior to being sorted using the automated cell deposition unit (ACDU)

of a FACStar^{PLUS} (Becton Dickinson, Sunnyvale, CA) flow cytometer. STRO-1^{bright}/VCAM-1+ cells were seeded at plating densities of 1, 2, 3, 4, 5, and 10 cells per well (96-well plates) in replicates of 24 wells per plating density (Figure 2). The cells were cultured in serum deprived medium on fibronectin coated wells as previously described [Gronthos and Simmons 1995; Gronthos *et al*, 1998]. On day 10 of culture the cells were then fixed and stained for 60 min with 0.1% toluidine blue in 1% paraformaldehyde. Aggregates of 50 cells were scored as CFU-F colonies and aggregates of 10<50 cells were scored as clusters using an Olympus SZ-PT dissecting light microscope (Olympus Optical Co. Ltd, Tokyo, Japan).

Analysis of cell cycling status of STRO-1+ BMMNC

The STRO-1$^+$ BMMNC were isolated by MACS as described above and then incubated with streptavidin PE for 15 min on ice. After washing twice with PBS the cells were fixed for 10 min with cold methanol (70%) on ice. Following this, the cells were washed three times with PBS and then incubated in blocking buffer for 15 minutes. The monoclonal antibody Ki-67 conjugated to FITC (DAKOPATTS A/S, Glostrup, Denmark) was added directly to the cells (1/10 dilution) in blocking buffer for 45 min on ice served as the negative control.

RNA Isolation and First-strand cDNA Synthesis

Total cellular RNA was routinely prepared from 2 x 10^4 STRO-1^{bright}/VCAM-1$^+$ cells collected as a bulk population and lysed using RNAzolB extraction method (Biotecx Lab. Inc., Houston, TX), as per manufacturers recommendations. RNA isolated from each subpopulation was then used as a template for cDNA synthesis. cDNA was prepared using a First-strand cDNA synthesis kit from Pharmacia Biotech (Uppsala, Sweden) according to manufacturers instructions. Briefly, total RNA was resuspended in 8 µl of DEPC-treated water and subsequently heated to 65°C for 10 min. Following snap cooling on ice, the RNA was added to 7 µl of premix containing reaction buffer, oligo-dT as primer and Superscript MMLV Reverse transriptase. Following incubation at 42°C for 60 min, the volume of the reaction was adjusted to 50µl with the addition of 35 µl of sterile water. The samples were stored at -20°C.

Polymerase chain reaction (PCR)

Due to limiting cell numbers, the expression of various bone-related transcripts (Table I) was assessed by polymerase chain reaction (PCR) amplification, using a standard protocol [Sambrook *et al*, 1989]. Two microlitres of first strand cDNA mixture from

each subpopulation was diluted in a 50µl PCR reaction (67mM Tris HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X100, 200µg/ml gelatin, 2mM $MgCl_2$, 200µM each dNTP) containing 100ng of each primer (Table 1), to which 2.5 units of Amplitaq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) was added. Reaction mixes were overlayed with mineral oil and amplification achieved by incubation in a Perkin-Elmer/Cetus thermal cycler. Primer design enabled typical cycling conditions of 94°C/(2 min), 60°C/(30 sec), 72°C/(1 min) for 40 cycles, with a final 10 min incubation at 72°C. To control for the integrity of the various RNA preparations, the expression of GAPDH and/or beta-2-microglobulin was also assessed. Following amplification, 10µl of each reaction mixture was analysed by 1.5% agarose gel electrophoresis, and visualised by ethidium bromide staining.

The developmental potential of BM CFU-F in vitro

We have previously reported the conditions for the induction of human bone marrow stromal cells to develop a mineralised bone matrix *in vitro* [Gronthos *et al*, 1994]. Briefly, the osteogenic and adipocytic potential of thirty day 4 CFU-F clones derived from single STRO-1^{bright}/VCAM-1^{+} sorted cells was assessed by culturing in alpha modification of Eagle's medium (α-MEM: Flow Laboratories) supplemented with 20% FCS, L-glutamine (2mM), β-mercaptoethanol (5 x 10^{-5} M), L-ascorbic acid 2-phosphate (100pM) (ASC-2P: Novachem, Melbourne, Australia), dexamethasone sodium phosphate (10^{-8}M) (DEX: David Bull Laboratories, Sydney, Australia), KH_2PO_4 (1.8 mM) (BDH Chemicals) and Hepes (10 mM), at 37°C, 5% CO_2. The media was changed twice a week for a period of six weeks. Cultures were rinsed twice with PBS then fixed in situ with 10% neutral formalin for 30 mon. Staining for vonKossa was performed according to the method of Pearse and Gardner (1972). Sections or culture wells were washed twice in distilled water and then stained in 5% aqueous $AgNO_3$ for 60 min under ultraviolet light. After staining with $AgNO_3$, the sections were washed twice with distilled water and then placed in 5% sodium thiosulphate for 1 min. Cultures were washed in distilled water, counter stained with Mayer's haematoxylin and mounted. Oil Red O (ORO) staining was performed as described by Grimble (1998). Briefly, cultures were fixed as described above, washed twice with PBS and air dried. Cultures were immersed in a solution 0.5% (w/w) ORO in isopropanol for 15 min at room temp., washed three times with distilled water and subsequently counterstained with haematoxylin.

Similarly, the chondrogenic potential of the same clones was assessed by culturing 2 x 10^5 cells per clone in 0.5 mls SDM supplemented with TGFβ1 and gently centrifuged

at 200g for 2 min in a 10 ml polypropolene tube then incubated at 37°C, 5% CO_2. The media was changed twice a week for a period of three weeks

The developmental potential of BM CFU-F in vivo

Bulk cultures of CFU-F derived from STRO-1^{bright}/VCAM-1^{+} sorted BMMNC were cultured for 5 weeks in the presence of ASC-2P and DEX and 10% FCS. The adherent cell layers were trypsinised and seeded onto 27mm^3 porous hydroxyapatite ceramic cubes (Zimmer Corporation, Warsaw, IN, USA) pre-coated with fibronectin (5µg/ml) (Boehringer Mannheim, Germany). The ceramic cubes were then implanted into subcutaneous pockets into the backs of SCID mice for a period of up to 8 weeks as described previously [Haynesworth *et al*, 1994; Kuznetsov *et al*, 1997]. Recovered implants were fixed in 10% buffered formalin for 2 days then decalcified for a further seven days in 0.5M EDTA before being embedded in paraffin wax. Cross-sections of the cubes were prepared as 5 µm sections onto glass slides pre-coated with Cell-Tak and counter stained with haematoxylin and eosin.

In situ *hybridization for the human specific alu sequence*

The HA ceramic implants were recovered 8 weeks post transplant and prepared for paraffin embedding on Cell-Tak coated slides as described above. To determine the origin of the cells within the implants *in situ* hybridization analysis was performed using a DNA probe specific to the unique human repetitive alu sequence [Kuznetsov *et al*, 1997]. The human specific alu sequence (pBLUR8; ATCC) was subcloned into the BamH1 restriction site of a pGEM-4Z plasmid (Promega). The digoxigennin-labeled alu specific probe was prepared by PCR containing 1 X PCR buffer (67 mM Tris HCl pH 8.8, 16.6 mM $(NH_4)_2S0_4$, 0.5% Triton-X100, 0.2 µg/ml gelatin, 2.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 1.9 mM dTTP, 0.1 mM digoxygenin-11-dUTP (Boehringer Mannheim), and 0.25 units of Amplitaq DNA Polymerase) and 100 ng of SP6 and T7 primers (Table 1) and 1 ng of plasmid DNA (pGEM-4Z; Promega Corp., Madison, WI) containing the alu sequence subcloned into the BamHI restriction site from (pBLUR8; ATCC, Rockville, MD). Sections were deparaffinized with xylene and ethanol then rehydrated through graded (100%, 90%, 70%, 50%) ethanol solutions. The sections were then treated with 0.2N for 7 min at room temperature and then incubated in 1 mg/ml pepsin (Sigma, St. Louis, MO) in 0.1N HCl for 10 minutes at 37°C. After washing in PBS, the sections were treated with 0.25% acetic acid containing 0.1M triethanolamine (pH 8.0) for 10 min and prehybridized with 50% deionized formamide containing 4X SSC for 15 min at 37°C. The hybridization solution (1 ng/µl digoxigenin-labeled probe in 1X Denhardt's

solution, 5% dextrane sulfate, 0.2mg/ml, salmon sperm DNA, 4X SSC, 50-% deionized formamide) was then added to the sections for denaturation at 95°C for 3 minutes followed by hybridization at 45°C for 3 hr. After washing with 2X SSC and 0.1X SSC, digoxigenin-labeled DNA was detected by immunohistochemistry using antidigoxigenin alkaline phosphatase-conjugated Fab fragments (1/5000; Boehringer Mannheim Corp., GMBH, Germany) followed by incubation with the corresponding alkaline phosphatase nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolyl-phosphate substrate solution as recommended by Boehringer Mannheim. Micrographs were taken with Ektachrome 64 T colour film using an Olympus IMT-2 inverted light microscope.

Telomerase Repeat Amplification Protocol (TRAP) Assay

Telomerase activity was measured by a modified non-radioactive TRAP protocol essentially as described by Fong *et al* (1997). Telomerase cell extracts were prepared by the method of Kim *et al,* (1994), with minor modifications. Populations of sorted or cultured cells were lysed in ice-cold CHAPS extraction buffer (0.5% 3[(3-cholamidopropyl)- dimethyl-ammonio]-1-propanesulfonate], 50 mM Tris-HCI, pH 7.4, 5 mM $MgCl_2$, 5 mM EGTA, 25 mM 2-mercaptoethanol, 1 ng/ml leupeptin, and 50% glycerol in DEPC-treated water), at a concentration of 1000 cells/µl, incubated on ice for 30 minutes and centrifuged at 16000 xg for 20 minutes at 4°C, the supernatant recovered and stored at -80°C until required. Detection of telomerase activity was performed in a two-step process as previously described (Fong *et al*, 1997). Briefly, to 2µl of cell extract, 16.5 µl of TRAP reaction buffer (20 mM Tris-HCI, pH8.2, 1.5mM $MgCl_2$, 63 mM KCl, 0.05%Tween-20, 1 mM EGTA), 100 ng of each of TS primer (5'- AATCCGTCGAGCAGAGTT-3'), and CX-ext primer (5'-GTGCCCTTCCCTTACCCTTACCC TAA-3'), 0.5 µL dNTPs (10 mM stock) were added, and the reaction mix incubated at 25°C for 30 minutes. Telomerase was subsequently inactivated by heating the reaction to 90°C for 2 minutes, prior to the addition of 5 µl of PCR mixture, containing 3.5 µl of TRAP reaction buffer, 1 µl of CX-ext primer and 2.5 U Taq polymerase. Reaction mixes were covered with mineral oil and placed in a Hybaid thermocycler, and subjected for 34 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 45 seconds, with a final extension at 72°C for 2 minutes. To confirm the specificity of the telomerase products, in all cases, a 2 µl aliquot of each CHAPS lysate was subjected to denaturation by heating samples at 100°C for 10 minutes. 25 µl of each reaction was resolved on a non-denaturing 12% polyacryalmide gel, and visualised by staining width SYBR Green fluorescent dye (FMC Bioproducts, OR, USA) as recommended by the manufacturer. The TRAP

products were analysed using a fluorescence scanning system (Molecular Dynamics, Sunnyvale, CA, USA).

Transmission Electron Microscopy (TEM)

STRO-1^{bright}/VCAM-1$^+$ cells (approximately 2 x 10^4 cells) were collected as a bulk population into eppendorf microtubes, washed once in 0.05M sodium cacodylate buffer and then fixed in 2.5% glutaraldehyde (EM Grade) in cacodylate buffer for 2 hr. The cultures were postfixed with 2% osmium tetroxide (VIII) (BDH Chemicals) in cacodylate buffer for 1 hr. After this, the cultures were dehydrated with graded ethanol solutions (70%, 90%, 100%). Epoxy resin (TAAB Laboratories; Berkshire, England) was then used to infiltrate the cultures overnight at 37°C. Polymerization of the resin was carried out at 60°C for 24 hr under vacuum. Ultrathin sections were cut on a LKB 8800 Ultrotome II (Broma, UK) and mounted onto copper grids. Sections were then examined using a JEOL 1200 EX II (Tokyo, Japan) transmission electron microscope. Photographs were taken using ILFORD EM Technical film.

RESULTS

Isolation and Purification of STRO-1$^+$ BM MPC

We have previously demonstrated the effectiveness of MACS to isolate and enrich for MPC from aspirates of human BM based on the cell surface expression of the STRO-1 antibody [Gronthos and Simmons, 1995; Gronthos *et al*, 1998]. In the present study, flow cytometric analysis of MACS isolated STRO-1$^+$ BMMNC cells demonstrated a heterogeneous pattern of expression spanning over four logs in fluorescence intensity (Figure. 1). Single-color FACS was subsequently employed to sort the STRO-1$^+$ BMMNC fraction into three subsets; STRO-1^{dull} STRO-1^{intermediate} and STRO-1^{bright}. Clonogenic assay for CFU-F in the different sorted STRO-1$^+$ subpopulations demonstrated that the majority of the MPC were contained within the STRO-1^{bright} cell fraction. There was a 900 fold increase in the incidence of CFU-F in the STRO-1^{bright} population when compared to unfractionated BMMNC (Table 1) demonstrating that BM MPC contained a high copy number of the STRO-1 antigen on their cell surface. The recovery of the MPC population in the STRO-1^{bright} fraction was >75% in respect to the estimated total number of CFU-F in the BM sample pre-MACS.

We attempted to obtain a more accurate discrimination of the STRO-1^{bright} subset by incubating the total STRO- 1$^+$ MACS isolated cells with the stromal cell surface antigen VCAM-1 (Figure. 2A) previously found to react exclusively with BM MPC [Simmons *et al*, 1994]. Dual color-FACS was used to identify and isolate the STRO-

1^{bright}/VCAM-1^+ BMMNC fraction. Limiting dilution analysis was subsequently performed, using the FACStar^{PLUS} automated cell deposition unit, to seed STRO-1^{bright}/VCAM-1^+ cells at various plating densities as described in the methods. Cells were cultured under serum deprived conditions in the presence of PDGF and EGF (10 ng/ml) previously found to support the clonogenic growth of CFU-F above that of serum replete conditions particularly at low plating densities [Gronthos and Simmons, 1995]. The mean incidence (n=6 different BM donors) of day 10 CFU-F colonies (>50 cells) was determined to be 1 CFU-F per 3 STRO1^{bright}/VCAM-1^+ cells plated using Poisson distribution statistics (Figure 2B). Furthermore, the incidence of clonogenic cells (clusters >10<50cells+ colonies) was found to be 1 per 2 STRO-1^{bright}/VCAM-1^+ cells plated (Figure. 2C). The MACS/FACS purlfication technique effectively achieved a 5 x 10^3 fold enrichment of the CFU-F population when compared to unfractionated BMMNC with an average incidence of 1 CFU-F colony per 10^4 BMMNC. It must also be stated that a proportion of the wells which were scored as 'negative' contained cell clusters of less than 10 cells.

Characterization of Purified BM MPC

Morphological examination of freshly sorted STRO-1^{bright}/VCAM-1^+ cells was carried out by transmission electron microscopy. Purified BM CFU-F appeared to be a homogeneous population of large cells containing many cytoplasmic processes and a large nucleous with an open chromatin structure (Figure. 3). To determine the cell cycling status of the CFU-F population in aspirates of BM the MACS isolated STRO-1^+ BMMNC fraction was further incubated with the cell cycling specific antigen Ki-67 [Gerdes *et al*, 1984; Wersto *et al*, 1988]. Two color flow cytometric analysis revealed that the STRO-1^{bright} subset which contained the CFU-F population lacked co-expression of the Ki-67 antigen demonstrating that these cells are non-dividing *in vivo* (Figure. 4A). Telomerase activity was examined in cell extracts from sorted and cultured candidate stromal progenitor cell populations by a modified TRAP assay. Telomerase activity was present in all fractions including the candidate stromal stem cell compartment isolated from adult bone marrow, defined by their expression of both the STRO-1 and VCAM-1 (CD106) cell surface molecules (Figure 4B).

To assess the proliferative capacity of BM MPC, individual CFU-F colonies (n=44) derived from two BM samples were expanded in the presence of serum under normal clonogenic growth conditions. A minor proportion of clones (8/44, 18%) demonstrated continued growth extending beyond 20 population doublings while the

remainder showed little or no proliferation beyond 12 population doublings (Figure 5). These cells also appeared to be capable of differentiating into adipose cells, whereas other isolated cells were less likely to do so.

A detailed phenotypic analysis of freshly isolated BM MPC pre-culture was compiled. Total RNA obtained from STRO-1^{bright}/VCAM-1^{+} cells was used to generate full-length first-strand cDNA as described in the methods. RT- PCR analysis revealed the presence of various bone cell markers including bonesialoprotein, osteonectin, and collagen type I. However, there was an absence in the expression of osteopontin, the parathyroid hormone receptor, and the more specific bone cell markers osteocalcin and the transcription factor CBFAI (Figure. 6A). Similarly, the fat-related markers lipoprotein lipase and the adipocyte human lipid binding protein were found to be expressed by the STRO-1^{bright}/VCAM-1^{+} population but there was no detectable expression of the adipocyte specific markers, the obese gene product (leptin) and the early transcription factor PPARγ2 in these cells (Figure. 6B). Furthermore the cartilage specific markers collagen type II and aggrecan were also not expressed by our purified MPC population. However the STRO-1^{bright}/VCAM-1^{+} cell fraction was found to express collagen type X, a marker associated with hypertrophic chondrocytes (Figure.6C). In addition, cytospin preparations of STRO-1^{bright}/VCAM-1^{+} sorted BMMNC failed to show any reactivity to the smooth muscle marker α-smooth muscle actin or with the endothelial marker, FVIII (data not shown). Overall the MPC population appeared to represent an early precursor population not yet fully commited to anyone particular stromal cell lineage.

Culture expanded bulk CFU-F derived from STRO-1^{bright}/VCAM-1^{+} sorted cells were assessed for their ability to develop into functional osteoblasts, chondrocytes and adipocytes *in vitro* as previously described [Gronthos *et al*, 1994]. A von Kossa positive mineralised matrix developed throughout the cultures by the end of the sixth week of induction (Figure. 7A). In addition, clusters of Oil Red O positive adipocytes were observed within the adherent layers in the same cultures (Figure. 7B). Following three weeks of chondrocytic induction in the presence of TGFβ1, the cells were also found to express the cartilage specific marker collagen type II by immunohistochemistry. Furthermore RT-PCR analysis of total RNA isolated from the different culture conditions demonstrated the expression of markers specific to bone (CBFA-1, OCN, PTH-R), fat (PPARγ2, leptin) and cartilage (collagen type II, aggrecan) (Figure. 6B).

The developmental potential of BM MPC Clones In Vitro and In Vivo

Bone marrow CFU-F clones were established from STRO-1^{bright}/VCAM-1^{+} sorted cells from three individual BM donors. At day 4 of culture, single clonogenic clusters were identified and expanded by subculture. Half of the cells from the first passage were taken from each clone and cultured under osteogenic growth conditions as described above. The osteogenic potential of ninety CFU-F clones was assessed where a von Kossa positive mineralised matrix formed in all of the ninety clones. However, only a proportion (38% ±15SEM, n=3) of the same clones gave rise to clusters of lipid containing oil red-O positive adipocytes demonstrating the bi-potential of the CFU-F population *in vitro*.

Half the cells from a representative 46 clones were subcultured and expanded for several weeks, then seeded into porous HA ceramic cubes and implanted subcutaneously into SCID mice for a period of 8 weeks as previously described [Haynesworth *et al*, 1992, Kusnetsov *et al*, 1997]. Cross-sections of the cubes prepared for histiological examination showed that all of the implants contained an extensive network of blood vessels and fibrous tissue (Figure 8A and Figure 8B). Bone formation was found in 42% (n=26) and 55% (n=20) of the clones isolated from two different BM aspirates. The ability of individual MPC clones to form a von Kossa positive mineralised matrix *in vitro* did not always correlate to the development of new bone *in vivo*. Similarly, the capacity of MPC clones to form adipocytic clusters *in vitro* had no bearing on the development of new bone formation *in vivo*.

The origin of the cellular material within the recovered implants was assessed by *in situ* hybridization using a DNA probe specific to the unique human repetitive alu sequence. The fibrous tissue, bone lining cells and osteocytes within the newly formed bone were all found to be positive for the alu sequence confirming their human origin and the bi-potential of a proportion of BM MPC (Figure9C and Figure 9D). Conversely, the fat and smooth muscle surrounding the ceramic cubes did not express the alu sequence and was therefore presumed to have originated from the host. Similarly, the endothelium lining the small blood vessels were also negative for the alu sequence implying they were derived from the mouse vasculature. In addition, there was no cartilage formation observed in sections of different implants and at different time points, as assessed by immunohistochemical analysis using a polyclonal antibody specific for collagen type II (data not shown).

USES OF MPCs

Example 1 - Repair of Articular Cartilage

Damaged articular cartilage generated by trauma or by diseases such as osteoarthritis and rheumatoid arthritis usually does not heal. However it is expected that this type of defect could be treated by implanting cultured MPCs of the present invention into the defect. The carrier may be pliable to mould to the shape of the defect and to promote round cell shape which is important for induction of chondrocyte differentiation. A suitable carrier may be constructed of collagen or fibrin. See Caplan *et al.* in US 5,226,914.

Example 2 - Repair of Bone

A combination of MPCs as well as a suitable support can be introduced into a site requiring bone formation. Cultured MPCs contained in calcium phosphate ceramic vehicles may be implanted into the defect site. For appropriate methods and techniques see Caplan *et al.* in US patent 5,226,914 and US patent 5,837,539.

Example 3 - Anchoring of Prosthetic Devices

The surface of a prosthetic device can be coated with MPCs prior to implantation. The MSCs can then differentiate into osteogenic cells to thereby speed up the process of bony ingrowth and incorporation of the prosthetic device. See Caplan *et al.* in US patent 5,226,914 and US patent 5,837,539.

Example 4 - Gene Therapy

An exogenous nucleic acid that encodes a protein or peptide with therapeutic may be transformed into the enriched population using standard techniques (see US patent 5591625 by Gerson *et al.*). The transformed cell population can then be introduced into the body of the patient to treat a disease or condition. For example, can be used to provide a continuous delivery of insulin, or genes encoding Factor VIII which is involved in clotting and therefore may be used in haemophiliacs.

Example 5 - Marrow Transplantation

A composition containing purified MPCs can be injected into a patient undergoing marrow transplantation prior to the introduction of the whole marrow. In this way the rate of haemopoiesis may be increased, particularly following radiation or chemotherapy. The composition might also include haemopoietic cells for use in radiotherapy or chemotherapy.

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CLAIMS

1. A method of enriching mesenchymal precursor cells, the method including the step of enriching for cells based on at least two markers, said markers being either:

a) the presence of markers specific for mesenchymal precursor cells, or

b) the absence of markers specific for differentiated mesenchymal precursor cells, or

c) the levels of expression of markers specific for differentiated mesenchymal cells.

2. A method of enriching mesenchymal precursor cells as in claim 1 wherein the method includes enriching by selecting for the positive expression of at least one of the markers.

3. A method of enriching mesenchymal precursor cells as in claim 2 wherein the method includes enriching by selecting for the positive expression of at least two of the markers.

4. A method of enriching mesenchymal precursor cells as in claim 3 wherein the markers are cell surface markers.

5. A method of enriching mesenchymal precursor cells as in claim 4 wherein the markers are selected from a group of surface markers specific for mesenchymal precursor cells including: LFA-3, THY-1, antigen identified by STRO-1, VCAM-1, ICAM-1, PECAM-1, P-selectin, L-selectin, CD49b/CD29, CD49c/CD29, CD49d/CD29, CD29, CD18, CD61, 6-19, thrombomodulin, CD10, CD13 and SCF.

6. A method of enriching mesenchymal precursor cells as in claim 5 wherein at least one of the markers is the antigen identified by STRO-1.

7. A method of enriching mesenchymal precursor cells as in claim 5 wherein at least one of the markers is VCAM-1.

8. A method of enriching mesenchymal precursor cells as in claim 5 wherein the two markers are the antigen identified by STRO-1, and VCAM-1.

9. A method of enriching mesenchymal precursor cells as in claim 4 wherein a proportion of the MPCs are capable of differentiation into at least two committed cell types selected from the group including adipose, areolar, osseous, cartilaginous, elastic and fibrous connective.

10. A method of enriching mesenchymal precursor cells as in claim 4 wherein the enrichment results in a cell population in which at least 1% of the cells are MPCs that are colony forming.

11. A method of enriching mesenchymal precursor cells as in claim 10 wherein the enrichment results in a cell population in which at least 5% of the cells are MPCs that are colony forming.

12. A method of enriching mesenchymal precursor cells as in claim 11 wherein the enrichment results in a cell population in which at least 10% of the cells are MPCs that are colony forming.

13. A method of enriching mesenchymal precursor cells as in claim 12 wherein the enrichment results in a cell population in which at least 40% of the cells are MPCs that are colony forming.

14. A method of enriching mesenchymal precursor cells as in claim 1 wherein the marker is the absence of cell surface markers indicative of commitment such as, CBFA-1, collagen type II, PPARγ2, glycophorin A.

15. A method of enriching mesenchymal precursor cells as in claim 4 wherein the method includes a first step of making a first partially enriched pool of cells by enriching for the positive expression of a first of the markers, and a second step of enriching for the positive expression of the second of the markers from the partially enriched pool of cells.

16. A method of enriching mesenchymal precursor cells as in claim 15 wherein the first step is a solid phase sorting step based on recognition of one or more of the markers, and the second step uses a more accurate separation method based on recognition of one or more of the markers, wherein the first step gives an enriched population with greater numbers of cells than if a high accuracy sorting step was used as a first step.

17. A method of enriching mesenchymal precursor cells as in claim 16 wherein the second step involves the use of two or more markers.

18. A method of enriching mesenchymal precursor cells as in claim 17 wherein the first step utilises MACS recognising expression of the antigen identified by STRO-1.

19. A method of enriching mesenchymal precursor cells as in claim 18 wherein the second sorting step utilises two colour FACS recognising expression of the antigen identified by STRO-1 as well as the expression of VCAM-1.

20. A method of enriching mesenchymal precursor cells as in claim 4 wherein recognition of cells carrying the cell surface markers is effected by binding a binding agent to the marker concerned followed by separation of those cells that exhibit binding, being either high level binding, low level binding or no binding.

21. A method of enriching mesenchymal precursor cells as in claim 20 wherein the binding agent is a monoclonal antibody or molecule based on a monoclonal antibody.

22. A method of enriching mesenchymal precursor cells as in claim 4 wherein the source of material for enrichment is stromal stem cells from one or more of the list including bone marrow, blood, epidermis and hair follicles.

23. A method of enriching mesenchymal precursor cells as in claim 22 wherein the source of material for enrichment is bone marrow.

24. A method of enriching mesenchymal precursor cells as in claim 4 wherein the method also includes the harvesting of a source of the stem cells before the enrichment step.

25. An enriched cell population wherein at least 1% of the cells are mesenchymal precursor cells that are colony forming.

26. An enriched cell population as in claim 25 wherein the cells carry at least two markers selected from a group of surface markers specific for mesenchymal precursor cells including LFA-3, THY-1, antigen identified by STRO-1, VCAM-1, ICAM-1,

PECAM-1, P-selectin, L-selectin, CD49b/CD29, CD49c/CD29, CD49d/CD29, CD29, CD18, CD61, 6-19, thrombomodulin, CD10, CD13 and SCF.

27. An enriched cell population as in claim 26 wherein the cells carry the antigen identified by STRO-1 and VCAM-1.

28. An enriched cell population wherein at least 5% of the cells are mesenchymal precursor cells that are colony forming.

29. An enriched cell population as in claim 28 wherein the cells carry at least two markers selected from a group of surface markers specific for mesenchymal precursor cells including LFA-3, THY-1, antigen identified by STRO-1, VCAM-1, ICAM-1, PECAM-1, P-selectin, L-selectin, CD49b/CD29, CD49c/CD29, CD49d/CD29, CD29, CD18, CD61, 6-19, thrombomodulin, CD10, CD13 and SCF.

30. An enriched cell population as in claim 29 wherein the cells carry the antigen identified by STRO-1 and VCAM-1.

31. An enriched cell population wherein at least 10% of the cells are mesenchymal precursor cells that are colony forming.

32. An enriched cell population as in claim 31 wherein the cells carry at least two markers selected from a group of surface markers specific for mesenchymal precursor cells including LFA-3, THY-1, antigen identified by STRO-1, VCAM-1, ICAM-1, PECAM-1, P-selectin, L-selectin, CD49b/CD29, CD49c/CD29, CD49d/CD29, CD29, CD18, CD61, 6-19, thrombomodulin, CD10, CD13 and SCF.

33. An enriched cell population as in claim 32 wherein the cells carry the antigen identified by STRO-1 and VCAM-1.

34. An enriched cell population wherein at least 40% of the cells are mesenchymal precursor cells that are colony forming.

35. An enriched cell population as in claim 34 wherein the cells carry at least two markers selected from a group of surface markers specific for mesenchymal precursor cells including LFA-3, THY-1, antigen identified by STRO-1, VCAM-1, ICAM-1,

PECAM-1, P-selectin, L-selectin, CD49b/CD29, CD49c/CD29, CD49d/CD29, CD29, CD18, CD61, 6-19, thrombomodulin, CD10, CD13 and SCF.

36. An enriched cell population as in claim 35 wherein the cells carry the antigen identified by STRO-1 and VCAM-1.

37. An enriched population of mesenchymal precursor cells as purified by the method of claim 1.

38. An enriched population of mesenchymal precursor cells as purified by the method of claim 8.

39. An enriched population of mesenchymal precursor cells as purified by the method of claim 19.

40. An enriched population of mesenchymal precursor cells as in either of claim 25 or claim 37 wherein a proportion of the mesenchymal precursor cells are capable of differentiation into at least two committed cell types selected from the group including adipose, areolar, osseous, cartilaginous, elastic and fibrous connective.

41. An enriched population of mesenchymal precursor cells as in either of claim 25 or claim 37 wherein the enriched population is suitable for seeding onto a vehicle for implantation to assist in bone growth.

42. An enriched population of mesenchymal precursor cells as in either of claim 25 or claim 37 wherein the enriched population has an exogenous nucleic acid transformed in to it so that the population may be introduced into the body of a patient to treat a disease or condition.

43. An enriched population of mesenchymal precursor cells as in either of claim 25 or claim 37 wherein the enriched population has an exogenous nucleic acid that expresses a therapeutic agent transformed in to it so that the population may be introduced into the body of a patient to release the therapeutic agent.

44. An enriched population of stem cells as in either of claim 25 or claim 37 wherein the enriched population is used to augment bone marrow transplantation.

45. A composition including the enriched population of claim 25.

46. A composition including the enriched population of claim 37.

47. A composition as in either of claim 45 or 46 wherein the composition is preadsorbed onto ceramic vehicles that are precoated with fibronectin and are suitable for implantation to augment bone marrow transplantation.

48. A composition as in either of claim 45 or 46 wherein the composition is suitable for use in augmenting bone marrow transplantation.

49. A composition as in claim 48 wherein the composition also includes haemopoietic cells.

50. A composition as in either of claim 45 or 46 wherein the population has an exogenous nucleic acid transformed in to it so that the composition may be introduced into the body of a patient to treat a disease or condition.

51. A composition as in either of claim 45 or 46 wherein the population has an exogenous nucleic acid that expresses a therapeutic agent transformed in to it so that the composition may be introduced into the body of a patient to release the therapeutic agent.

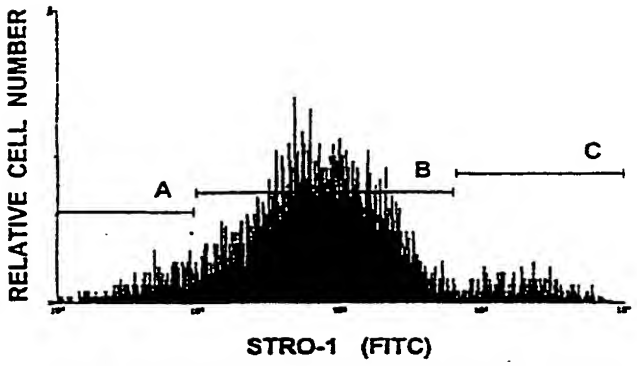

FIGURE 1

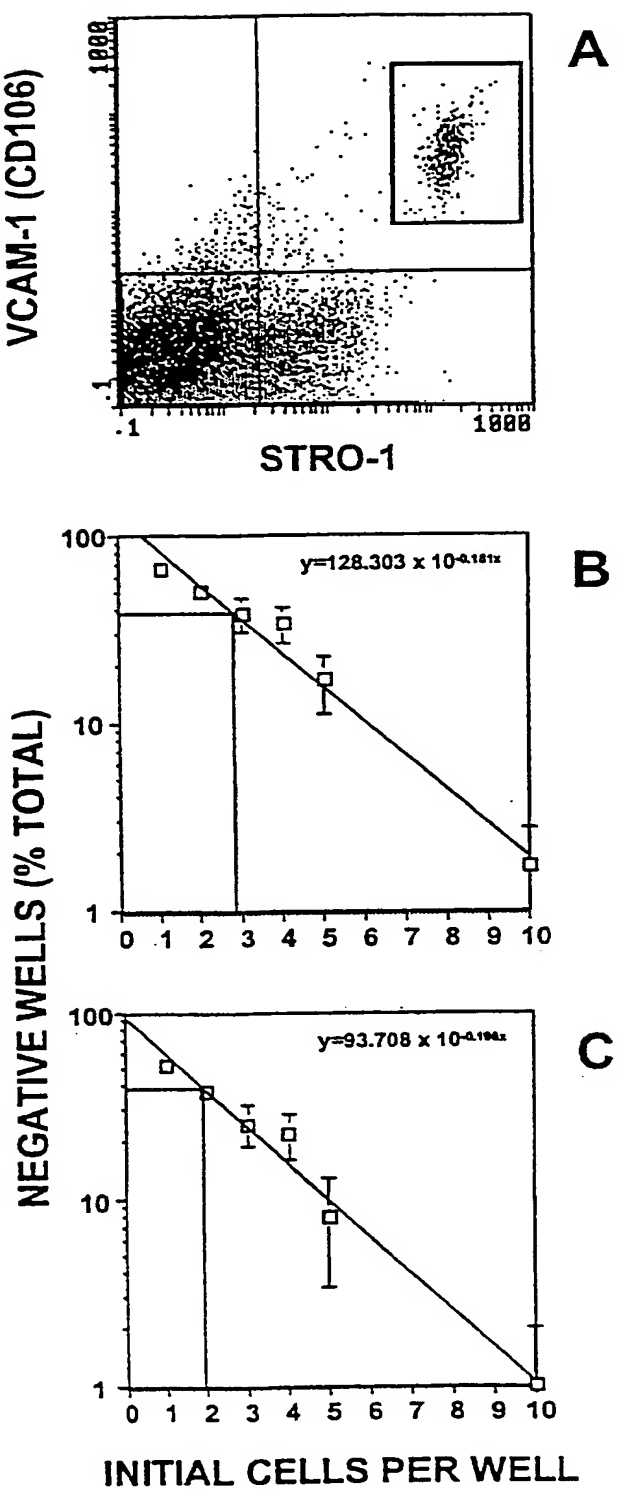
A
VCAM-1 (CD106)
1000
.1
.1
1000
STRO-1
B
y=128.303 x 10^-0.161x
100
10
1
0 1 2 3 4 5 6 7 8 9 10
C
y=93.708 x 10^-0.196x
100
10
1
0 1 2 3 4 5 6 7 8 9 10
NEGATIVE WELLS (% TOTAL)
INITIAL CELLS PER WELL

FIGURE 2

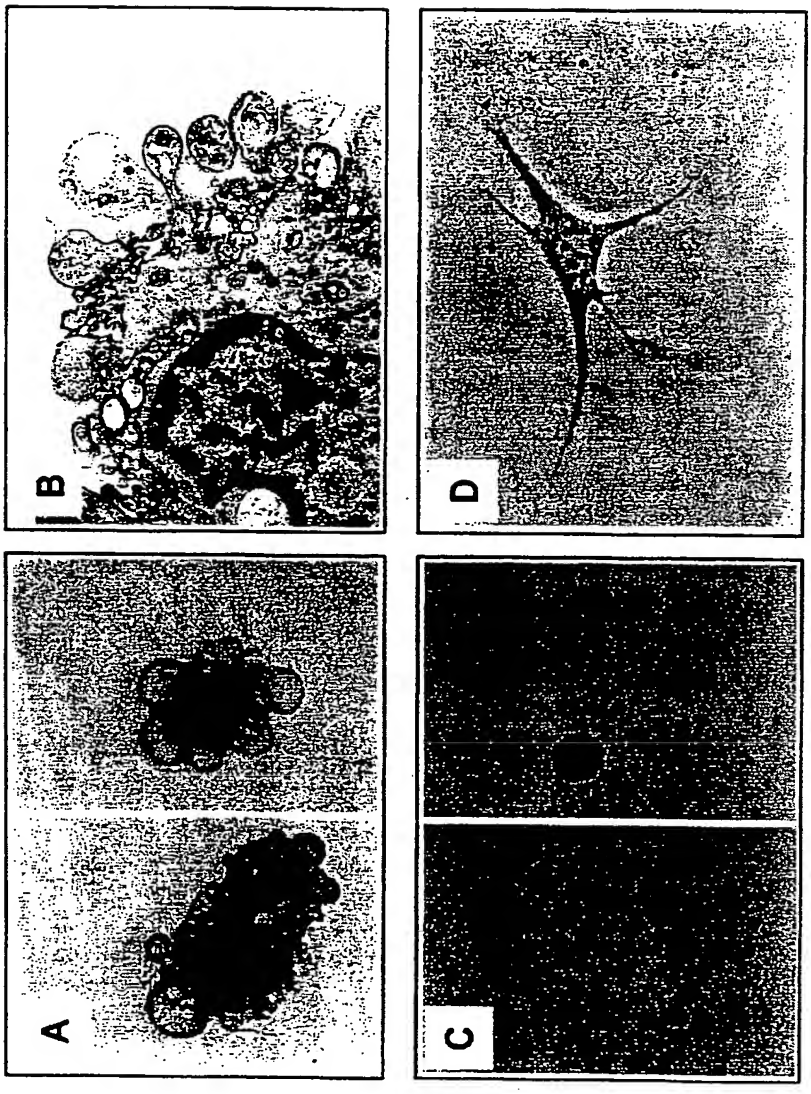

FIGURE 3

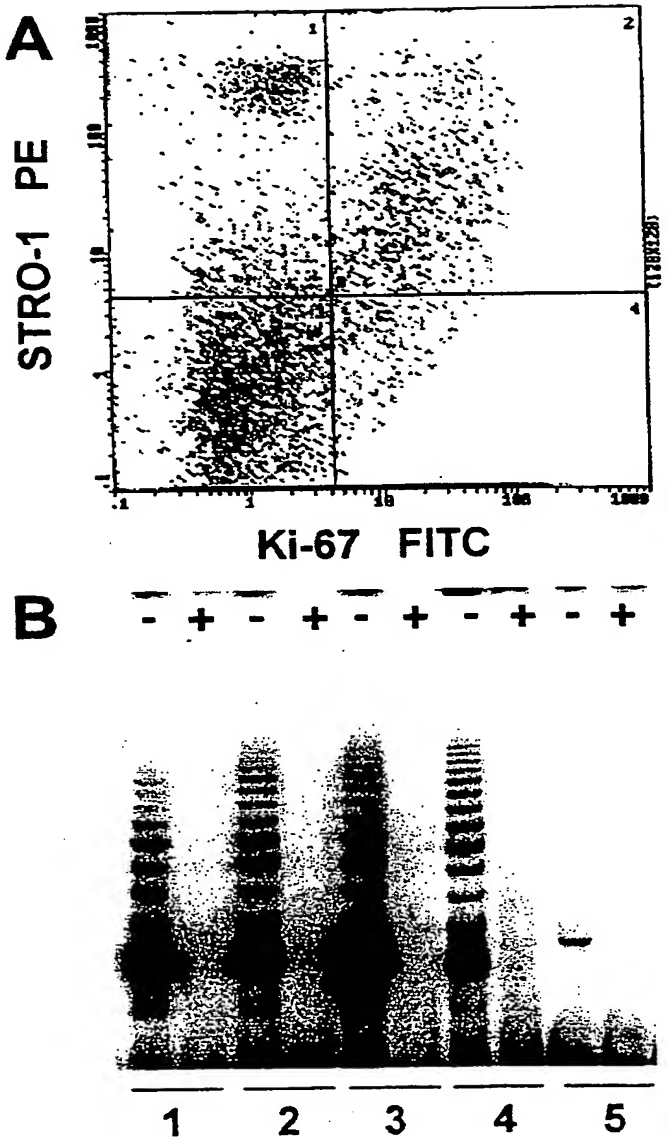

FIGURE 4

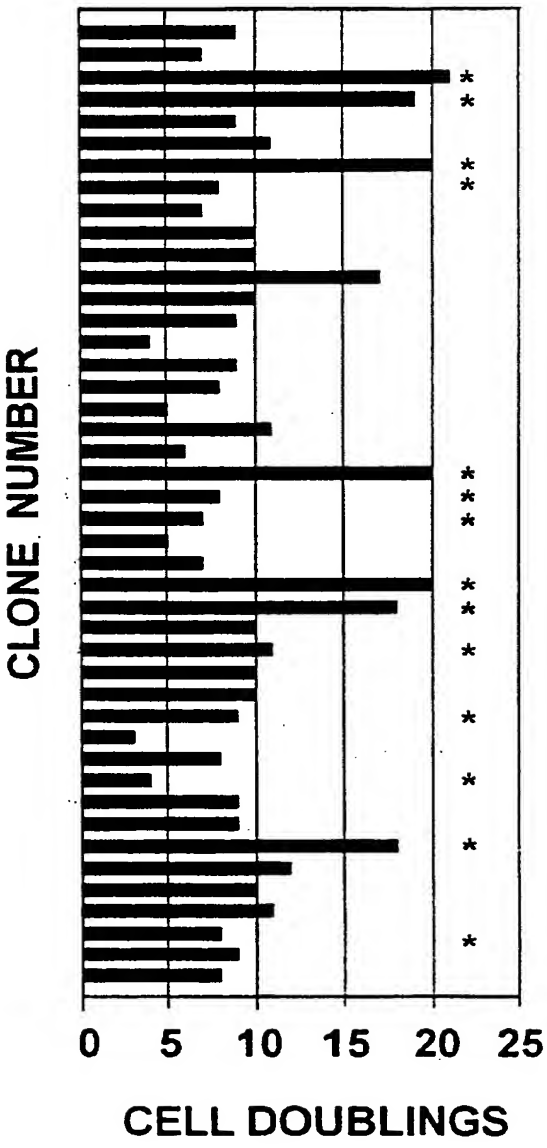

FIGURE 5

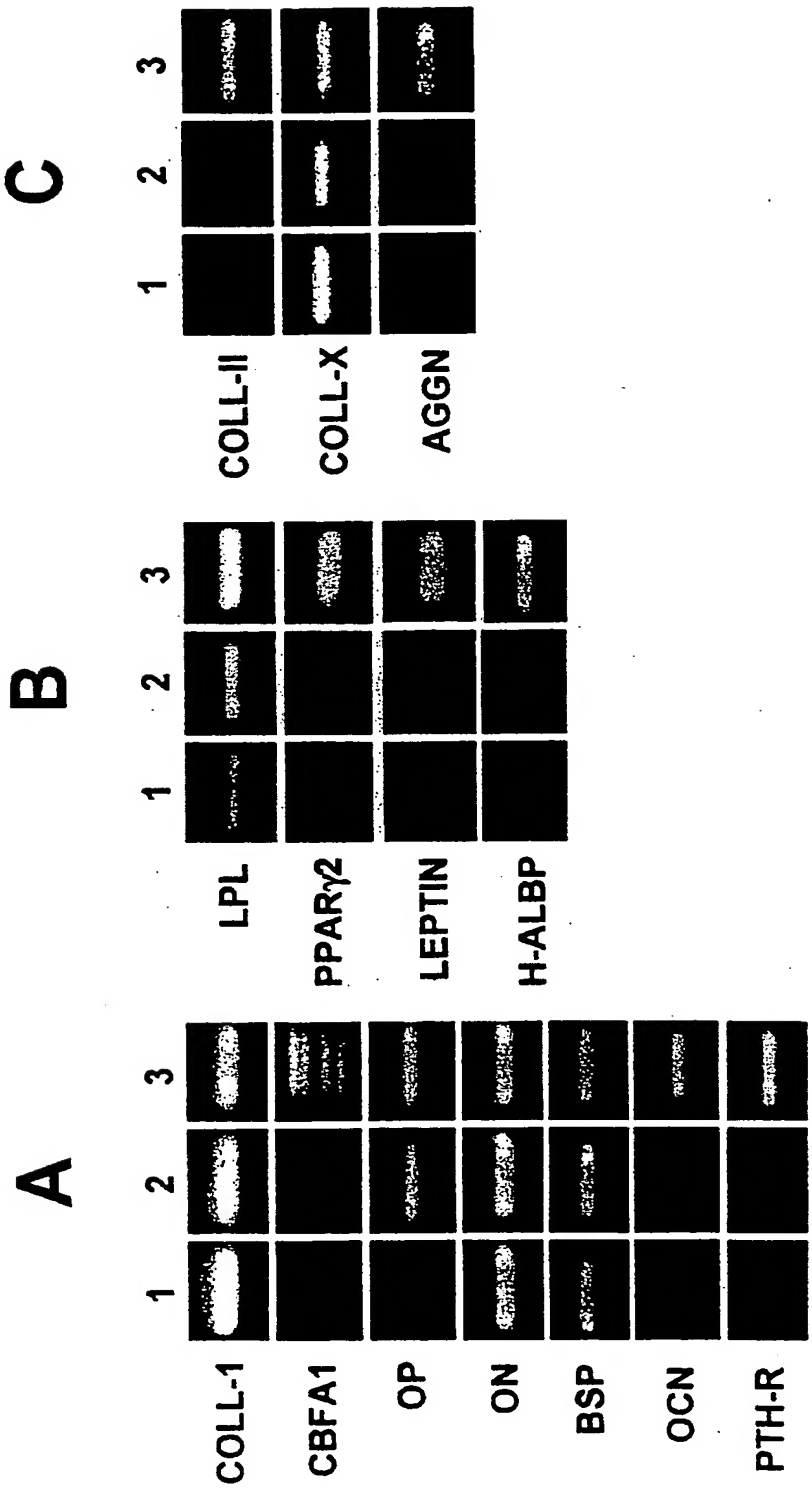
A
1
2
3
COLL-1
CBFA1
OP
ON
BSP
OCN
PTH-R
B
1
2
3
LPL
PPARγ2
LEPTIN
H-ALBP
C
1
2
3
COLL-II
COLL-X
AGGN

FIGURE 6

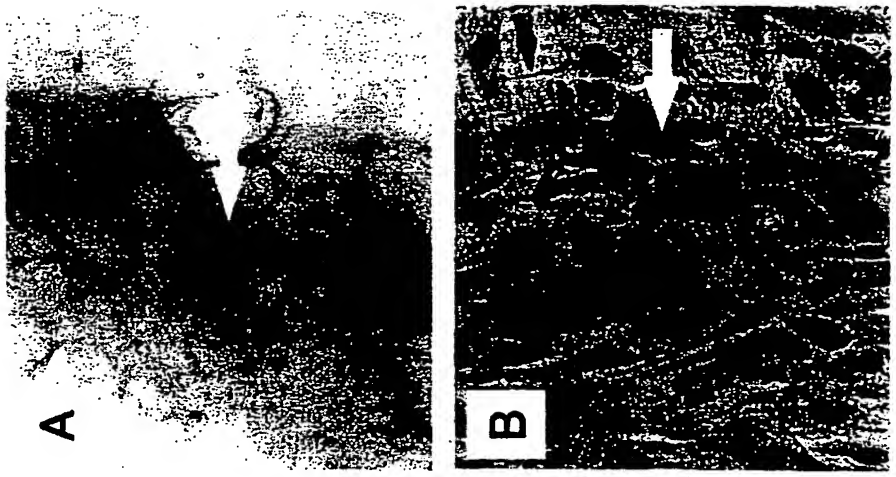

FIGURE 7

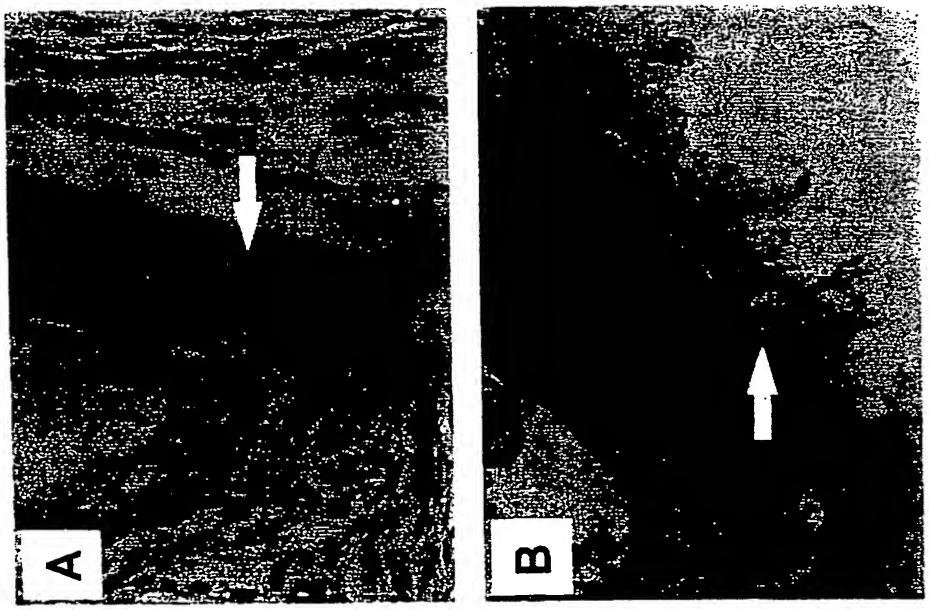

FIGURE 8

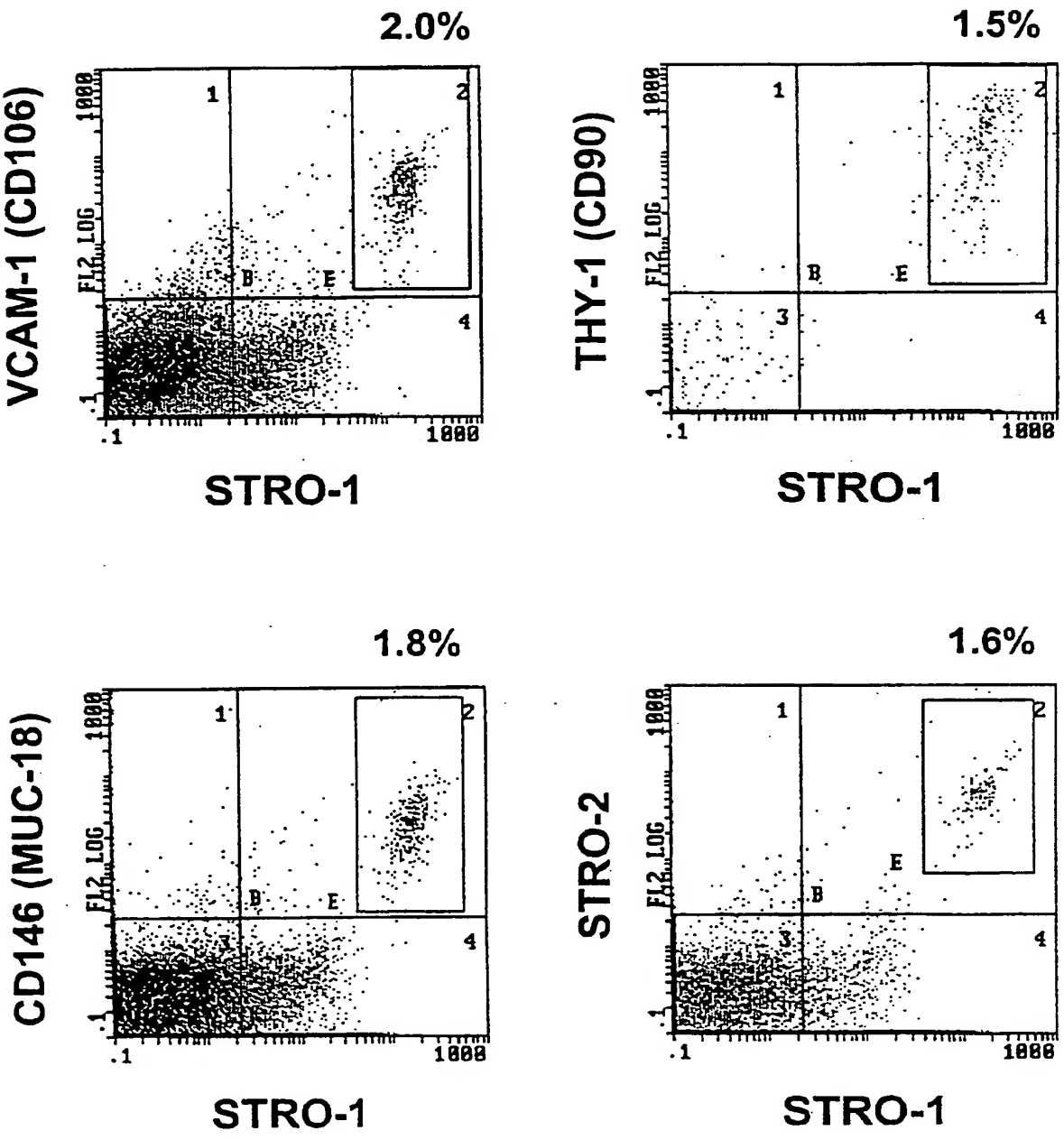
2.0%
VCAM-1 (CD106)
FL2 LOG
STRO-1
1.5%
THY-1 (CD90)
FL2 LOG
STRO-1
1.8%
CD146 (MUC-18)
FL2 LOG
STRO-1
1.6%
STRO-2
FL2 LOG
STRO-1

FIGURE 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00822

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 005/00, 005/08,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Chem Abs, Medline, WPIDS: enrich?, mesenchym?, purif?,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SIMMONS, P. J. et al (1994) "Isolation, characterisation and functional activity of human marrow stromal progentors in hemopoiesis" *Advances in Bone Marrow Purging and Processing: Fourth International Symposium*, pages 271-280.	
A	GRONTHOS, S. et al (1994) "The STRO-1$^+$ Fraction of Adult Human Bone Marrow Contains the Osteogenic Precursors" *Blood*, vol. 84, No. 12, pages 4164-4173.	

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

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"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2000	- 8 AUG 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **PHILIPPA WYRDEMAN** Telephone No : (02) 6283 2554

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(25) Filing Language: English

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(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
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Filed on 8 July 1999 (08.07.1999)

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(72) Inventors; and
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(54) Title: A LUNG CANCER ASSOCIATED RETROVIRUS, GENE DELIVERY VECTOR AND METHODS OF USE THEREOF

(57) Abstract: A viral genomic sequence of Jaagsiekte sheep retrovirus (JSRV) is provided and characterized herein. Also provided are methods of using the JSRV in gene therapy and in the treatment and diagnosis of JSRV related disorders.

A LUNG CANCER ASSOCIATED RETROVIRUS, GENE DELIVERY VECTOR AND METHODS OF USE THEREOF

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

The U.S. Government may have certain rights in this invention pursuant to Grant No.: RO1CA82564 by the National Institute of Health (NIH).

FIELD OF THE INVENTION

The present invention relates generally to the field of virology and more specifically to a novel retrovirus useful for the transfer and expression of nucleic acid sequences in a targeted cell and methods of diagnosing and treating disease associated with retroviruses.

BACKGROUND

Sheep pulmonary adenomatosis (SPA) is a contagious and experimentally transmissible lung cancer of sheep resembling human bronchiolo-alveolar carcinoma. A type D retrovirus, known as jaagsiekte sheep retrovirus (JSRV), has been associated with the etiology of SPA, but its exact role in the induction of the tumor has not been clear due to the lack of (i) a tissue culture system for the propagation of JSRV and (ii) an infectious JSRV molecular clone.

Animal models of retrovirus-induced tumors have provided many insights into the mechanisms governing cell transformation (Vogt, P. K. 1997. p. 1-25. *In* J. M. Coffin, S. H. Hughes, and H. E. Varmus (ed.), Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Sheep pulmonary adenomatosis (SPA), also known as ovine pulmonary carcinoma, is a bronchiolo-alveolar carcinoma that is present in widely distributed agricultural populations (Hecht *et al.* 1996. Br. Vet. J. 152:395-409; Palmarini *et al.* 1997. Trends Microbiol. 5:478-483). SPA strongly resembles human bronchiolo-alveolar carcinoma (BAC); both tumors have the same clinical, macroscopic, histopathologic, and ultrastructural features (Ives *et al.* 1983. Am. Rev. Respir. Dis. 128:195-209; Perk, K., and I. Hod. 1982. JNCI 69:747-749). BAC has many pathological and epidemiological characteristics that

distinguish it from other types of human lung cancer, including adenocarcinoma (Barkley *et al.* 1996 J. Clin. Oncol. 14:2377-2386; Carney and L. De Leij. 1988. Semin. Oncol. 15:199-214; Clayton, F. 1988. Pathol. Annu. 23:361-394). The incidence of BAC is rising, and now represents up to a quarter of primary lung cancers in the United States (Barsky *et al.* 1994. Cancer 73:1163-1170). Most notably, lung cancer is the main cause of death from cancer in both men and women (Landis *et al.* 1998. CA Cancer J. Clin. 48:6-29; Wingo *et al.* 1998. Cancer 82:1197-1207), but very few animal models are available. The common characteristics between human BAC and SPA suggest that SPA could be a unique experimental model and could offer novel insights into pulmonary carcinogenesis.

SPA also is a significant veterinary problem in countries such as the United Kingdom, South Africa, and Spain. The cumulative lifetime risk for developing SPA approaches 25% in high-risk flocks in these countries (Sharp and K. Angus. 1990. p. 177-185. *In* G. Petursson, and R. Hoff-Jogensen (ed.), Maedi-Visna and related diseases. Kluwer Academic Publishers, Boston, Mass.).

Previous experiments provided evidence for the presence of a retrovirus (jaagsiekte sheep retrovirus (JSRV)) in the tumors and lung secretions of SPA-affected sheep (Hecht *et al.* 1994. Virology 202:480-484; Martin *et al.* 1976. Nature 264:183-185; Rosadio *et al.* 1988. Vet. Pathol. 25:475-483; Sharp, J. M., and A. J. Herring. 1983. J. Gen. Virol. 64:2323-2327). An important development was the deduction of a nucleotide sequence of a South African strain of JSRV (JSRV-SA) (York *et al.* 1992. J. Virol. 66:4930-4939; York *et al.* 1991. J. Virol. 65:5061-5067). This was accomplished by piecing together cDNA clones and reverse transcriptase PCR (RT-PCR) products from a cDNA library constructed from DNA isolated by isopycnic centrifugation from the lung fluid of an SPA-affected animal. However, competent viral particles have been unable to be developed using cells transfected with this sequence.

Despite the recent data strongly suggesting that JSRV is the cause of SPA, a reconstructed JSRV-SA provirus failed to reproduce SPA in sheep. Therefore, it has been unclear if JSRV is alone sufficient to induce lung cancer in sheep, if it is a helper

virus for an unidentified acutely transforming retrovirus, or if it simply is a passenger that replicates preferentially in SPA tumor cells.

SUMMARY OF THE INVENTION

The invention provides an isolated replication competent infectious Jaagsiekte sheep retrovirus (JSRV). In one embodiment, the invention provides an isolated retrovirus having a JSRV GAG protein; a JSRV POL protein; a JSRV ENV protein; a JSRV genome comprising Long-Terminal Repeat (LTR) sequences at the 5' and 3' end of the retroviral genome, wherein the LTR is active in pulmonary epithelial cells, a polynucleotide sequence encoding JSRV GAG protein, JSRV POL protein, and JSRV ENV protein; and cis-acting nucleic acid sequences necessary for reverse transcription, packaging and integration in a target cell. In another embodiment, the JSRV has a genomic sequence as set forth in GenBank accession no. AF105220.

The invention also provides an isolated Jaagsiekte sheep retrovirus (JSRV) genome, having a polynucleotide sequence as set forth in GenBank accession no. AF105220.

In yet another embodiment, the invention provides a method for producing an infectious Jaagsiekte sheep retrovirus (JSRV). The method includes transfecting a host cell with the vector containing a polynucleotide genome of JSRV, culturing the host cell under sufficient conditions and for sufficient time to allow expression of the vector to produce JSRV viral particles; and obtaining the JSRV viral particles.

In another embodiment, the invention provides a method of treating a subject having a cell proliferative disorder or a biochemical or genetic defect. The method includes contacting the subject with a retroviral vector, comprising, a JSRV GAG protein; a JSRV POL protein; a JSRV ENV protein; a JSRV genome comprising Long-Terminal Repeat (LTR) sequences at the 5' and 3' end of the retroviral genome, wherein the LTR is active in pulmonary epithelial cells, a heterologous nucleic acid sequence operably linked to a regulatory nucleic acid sequence; and cis-acting nucleic

acid sequences necessary for reverse transcription, packaging and integration in a target cell.

The invention also provides a pharmaceutical composition useful for inducing an immune response to Jaagsiekte sheep retrovirus (JSRV) in an subject. The composition includes an immunogenically effective amount of a JSRV or JSRV polypeptide in a pharmaceutically acceptable carrier.

In another embodiment, a method of inducing an immune response to a JSRV or JSRV polypeptide in a subject is provided. The method includes immunizing an animal with the composition a pharmaceutical composition useful for inducing an immune response to Jaagsiekte sheep retrovirus (JSRV) in an subject. The composition includes an immunogenically effective amount of a JSRV or JSRV polypeptide in a pharmaceutically acceptable carrier.

In another embodiment, the invention provides an antibody which specifically binds to the replication competent infectious Jaagsiekte sheep retrovirus (JSRV).

In yet another embodiment, the invention provides a method for inhibiting the binding of a JSRV to a cell comprising contacting the JSRV with an anti- JSRV-antibody.

The invention also provides a method for identifying a compound which binds to a Jaagsiekte sheep retrovirus (JSRV). The method includes incubating components comprising the compound and the JSRV under conditions sufficient to allow the components to interact; and measuring the binding or effect of binding of the compound to the JSRV. In one embodiment, the effect can be detected by measuring the infectivity of JSRV.

Also provided is a method for inhibiting the expression of Jaagsiekte sheep retrovirus (JSRV) in a cell comprising contacting the cell with an inhibiting effective amount of an antisense oligonucleotide that binds to a segment of an mRNA transcribed from the JSRV genome whereby the binding of the antisense to the mRNA segment inhibits JSRV gene expression.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the cloning of $JSRV_{21}$. The strategy used for the isolation of $JSRV_{21}$ is shown

FIG. 2 shows $JSRV_{21}$-based plasmid constructs and *in vitro* synthesis of viral particles. (a) Schematic representation of the genomic organization of the $JSRV_{21}$ provirus; standard retroviral notation is used. The proviral genome is typical of type B and type D retroviruses, with *pro* in a different open reading frame from *pol*. Note the presence of an accessory open reading frame (*orf-x*) overlapping *pol*. (b) $pJSRV_{21}$ and $pCMV2JS_{21}$ plasmid constructs. In $pCMV2JS_{21}$, the U3 region of the proximal LTR was replaced by the human CMV promoter.

FIG. 3 shows the buoyant-density analysis of $JSRV_{21}$. (a) Intact $JSRV_{21}$ particles prepared by transient transfection of 293T cells with $pCMV2JS_{21}$ were analyzed by isopycnic centrifugation in a 25 to 60% (wt/wt) sucrose gradient. Adjacent fractions were pooled, and exogenous RT activity was determined (solid lines). The densities of each fraction are shown in grams per milliliter (dashed lines). (b) $JSRV_{21}$ was treated with 0.1% Triton X-100 and analyzed as in panel a.

FIG. 4 shows a schematic representation of the genomic structure of the type D retroviruses of sheep. Premature stop codons are indicated by a vertical bar underlined by an asterisc. For convenience the *gag* open reading frame has been fixed at position –3 in all the sequences shown. The numbered bar at the bottom indicate distances in kilobases. The exogenous JSRV and ENTV show the canonical retroviral *gag*, *pro*, *pol* and *env* with *pro* in a different open reading frame from *pol* as in all type D and B retroviruses. An additional open reading frame (*orf-x*) overlapping *pol* is present in JSRV but it is interrupted by two stop codons in ENTV. enJS56A1 is the only one of the three endogenous loci cloned in this study to maintain full open reading frames in all the structural genes. EnJS59A1 has premature stop codons in *gag* and *pol* and an ample deletion in *env*. enJS5F16 has a deletion in *pol*.

FIG. 5 shows the alignment of the deduced amino acid sequence of *gag* of type D retrovirus of sheep. Alignment is shown of the exogenous $JSRV_{21}$

(AF105220), JSRV-SA (M80216), ENTV (Y16627) and the endogenous en5F16 and en56A1 that maintain an open reading frame along the whole *gag*. Dots refer to identical sequences while dashes indicate lack of sequence. Underlined are the variable region A and B (VRA and VRB). Note the proline rich region present in the VRA of the exogenous JSRVs and ENTV that is instead absent in the endogenous loci. In VRB, ENTV is more similar to JSRV than *enJSRVs*. Indicated is the putative major capsid region (CA) and the *HpaI* site used to generate exogenous-endogenous chimeras.

FIG. 6 shows the alignment of the deduced amino acid sequence of *env* of type D endogenous retroviruses of sheep. Alignment is shown of the exogenous $JSRV_{21}$ (AF105220), JSRV-SA (M80216), ENTV (Y16627) and the endogenous en5F16 and en56A1 that maintain an open reading frame along the whole *env*. Dots refer to identical sequences while dashes indicate lack of sequence. The boundaries between the surface (SU) and transmembrane (TM) are indicated. The variable region C (VRC) is underlined. Note the polymorphism between all the sequences in VRC.

FIG. 7 shows the construction of endogenous-exogenous chimeras for the identification of the enJS56A1 packaging defect. Schematic structure of the parental ($JSRV_{21}$ and enJS56A1) and chimeric plasmids. The restriction enzyme sites used for the cloning procedure are indicated. In pCMV2JS21 and in the various chimeric constructs expression is driven by the cytomegalovirus immediate-early promoter (CMV, indicated by an arrow).

FIG. 8 shows the alignment of the nucleotide sequence of the untranslated *gag* region of type-D retroviruses of sheep. Alignment is shown of the exogenous $JSRV_{21}$ (AF105220), JSRV-SA (M80216), ENTV (Y16627) and the endogenous en5F16, en59A1 and en56A1. The primer binding site (PBS) is underlined.

FIG. 9 shows a phylogenetic analysis of the type D retroviruses of sheep. Unrooted phylogenetic trees for the U3 (A), *env* (B) and *gag* and *pol* (C) has been estimated by neighbor joining. To show consistency all bootstrap values obtained with 1,000 replications of bootstrap sampling are shown. Sequences used for the analysis

are termed as in its original reference with the exception of Locus 1-6 which are indicated L1-L6 in (A). The GenBank accession numbers are: AF105220 ($JSRV_{21}$); M80216 (JSRV-SA); X95445-X95452 (endogenous Locus 1, 2, 3, 4, 5, 6 and exogenous type I and II LTR); Y16627 (ENTV); Y18301-Y18305 (JS7, 809T, 83RS28, 92K3); Z66531-Z66533 (enJSRV1, 2, 3); Z71304 (LTR-UK); (AF136224) enJS5F16; (AF136225) enJS59A1; AF153615 (enJS56A1). In all the estimated trees there are 5 distinct phylogenetic groups for the type-D retroviruses: *enJSRV-A*, *enJSRV-B* for the endogenous loci; the ENTV group and two groups for the exogenous JSRV, JSRV-I (African isolates) and JSRV-II (isolates from USA and UK).

FIG. 10 shows enJSRVs LTR activity in cell lines. The penJS56A1-luc, penJS5F16-luc and penJS59A1-luc plasmids were transfected into various cell lines as described herein. Cell lines used were derived from mouse differentiated lung epithelial cells (MLE-15 and mtCC1-2) and extrapulmonary tissues such as mouse fibrobalsts (NIH-3T3), mouse kidney (TCMK) and sheep endometrium (LE). Luciferase activities of the various endogenous loci LTR relative to the activity of pJS21-luc, a reporter plasmid driven by the $JSRV_{21}$ LTR are shown. The activity of pJS21-luc in each cell line was set at 100 percent. Results shown are the average of 6-12 replicates.

FIG. 11 shows the transactivation of JSRV and *enJSRVs* LTR by HNF-3α and HNF-3β. pJS21-luc, penJS56A1-luc, penJS5F16-luc and penJS59A1-luc were co-transfected into NIH-3T3 cells (that do not efficiently support JSRV enhancer activity) along with expression plasmids for either HNF-3α or β. Different amounts of the transcription factor expression plasmids were co-transfected with a set amount (200ng) of the reporter plasmid DNA. The amounts of luciferase activity for the different co-transfections are shown as fold activation of the reporter plasmid transfected with a plasmid having the CMV promoter but no HNF-3 insert.

DETAILED DESCRIPTION OF THE INVENTION

A novel viral genomic sequence of Jaagsiekte sheep retrovirus (JSRV) has been isolated and characterized herein. Exogenous JSRV sequences are present in the tumor tissues of SPA-affected (or experimentally infected) sheep but not in unaffected animals. Normal sheep have 15 to 20 copies of JSRV-related endogenous retroviruses, some of which are transcriptionally active. The tumor cells from the lungs of SPA-affected sheep are the main sites of JSRV replication, but viral DNA and RNA also can be detected in various lymphoid tissues, where the virus appears to infect a wide variety of lymphoid cell.

SPA strongly resembles human bronchiolo-alveolar carcinoma (BAC); both tumors have the same clinical, macroscopic, histopathologic, and ultrastructural features. BAC has many pathological and epidemiological characteristics that distinguish it from other types of human lung cancer, including adenocarcinoma. The incidence of BAC is rising, and it now represents up to a quarter of primary lung cancers in the United States. Most notably, lung cancer is the main cause of death from cancer in both men and women, but very few animal models are available. The common characteristics between human BAC and SPA suggest that SPA could be a unique experimental model and could offer novel insights into pulmonary carcinogenesis. SPA also is a significant veterinary problem in countries such as the United Kingdom, South Africa, and Spain. The cumulative lifetime risk for developing SPA approaches 25% in high-risk flocks in these countries.

To explore the role of JSRV in the etiology of SPA, and to identify related activities in BAC, the inventors have succeeded in molecularly cloning a JSRV provirus and assessed the infectivity and pathogenicity of this clone *in vivo*. The results established that JSRV is necessary and sufficient for induction of SPA. In addition, the invention provides a culture system for the production of JSRV and recombinant JSRV, as well as vaccines, diagnostics and recombinant vectors using JSRV. SPA represents a unique model for lung cancer, and studies on its aetiopathogenesis can provide further insight into the mechanisms of epithelial neoplasms.

Retroviruses are RNA viruses wherein the viral genome is RNA. When a host cell is infected with a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate which is integrated efficiently into the chromosomal DNA of infected cells. The integrated DNA intermediate is referred to as a provirus. The family Retroviridae are enveloped single-stranded RNA viruses that typically infect mammals, such as, for example, bovines, monkeys, sheep, and humans, as well as avian species. Retroviruses are unique among RNA viruses in that their multiplication involves the synthesis of a DNA copy of the RNA which is then integrated into the genome of the infected cell.

The Retroviridae family consists of three groups: the spumaviruses (or foamy viruses) such as the human foamy virus (HFV); the lentiviruses, as well as visna virus of sheep; and the oncoviruses (although not all viruses within this group are oncogenic). The term "lentivirus" is used in its conventional sense to describe a genus of viruses containing reverse transcriptase. The lentiviruses include the "immunodeficiency viruses" which include human immunodeficiency virus (HIV) type 1 and type 2 (HIV-1 and HIV-2) and simian immunodeficiency virus (SIV). The oncoviruses are further subdivided into groups A, B, C and D on the basis of particle morphology, as seen under the electron microscope during viral maturation. A-type particles represent the immature particles of the B- and D-type viruses seen in the cytoplasm of infected cells. These particles are not infectious. B-type particles bud as mature virion from the plasma membrane by the enveloping of intracytoplasmic A-type particles. At the membrane they possess a toroidal core of ~75 nm, from which long glycoprotein spikes project. After budding, B-type particles contain an eccentrically located, electron-dense core. The prototype B-type virus is mouse mammary tumor virus (MMTV). No intracytoplasmic particles can be observed in cells infected by C-type viruses. Instead, mature particles bud directly from the cell surface via a crescent "C"-shaped condensation which then closes on itself and is enclosed by the plasma membrane. Envelope glycoprotein spikes may be visible, along with a uniformly electron-dense core. Budding may occur from the surface plasma membrane or directly into intracellular vacuoles. The C-type viruses are the most commonly studied and include many of the avian and murine leukemia viruses (MLV). Bovine leukemia virus (BLV), and the human T-cell leukemia viruses types I

and II (HTLV-I/II) are similarly classified as C-type particles because of the morphology of their budding from the cell surface. However, they also have a regular hexagonal morphology and more complex genome structures than the prototypic C-type viruses such as the murine leukemia viruses (MLV). D-type particles resemble B-type particles in that they show as ring-like structures in the infected cell cytoplasm, which bud from the cell surface, but the virion incorporate short surface glycoprotein spikes. The electron-dense cores are also eccentrically located within the particles. Mason Pfizer monkey virus (MPMV) is the prototype D-type virus.

Retroviruses are defined by the way in which they replicate their genetic material. During replication the RNA is converted into DNA. Following infection of the cell a double- stranded molecule of DNA is generated from the two molecules of RNA which are carried in the viral particle by the molecular process known as reverse transcription. The DNA form becomes covalently integrated in the host cell genome as a provirus, from which viral RNAs are expressed with the aid of cellular and/or viral factors. The expressed viral RNAs are typically packaged into particles and released as infectious virion.

The retrovirus particle is composed of two identical RNA molecules. Each wild-type genome has a positive sense, single-stranded RNA molecule, which is capped at the 5' end and polyadenylated at the 3' tail. The diploid virus particle contains the two RNA strands complexed with gag proteins, viral enzymes (*pol* gene products) and host tRNA molecules within a "core" structure of gag proteins. Surrounding and protecting this capsid is a lipid bilayer, derived from host cell membranes and containing viral envelope (env) proteins. The env proteins bind to a cellular receptor for the virus and the particle typically enters the host cell via receptor-mediated endocytosis and/or membrane fusion.

After the outer envelope is shed, the viral RNA is copied into DNA by reverse transcription. This is catalyzed by the reverse transcriptase enzyme encoded by the pol region and uses the host cell tRNA packaged into the virion as a primer for DNA synthesis. In this way the RNA genome is converted into a DNA genome.

The double-stranded linear DNA produced by reverse transcription may, or may not, have to be circularized in the nucleus. The provirus now has two identical repeats at either end, known as the long terminal repeats (LTR). The termini of the two LTR sequences produces the site recognized by a pol product - the integrase protein - which catalyzes integration, such that the provirus is always joined to host DNA two base pairs (bp) from the ends of the LTRs. A duplication of cellular sequences is seen at the ends of both LTRs, reminiscent of the integration pattern of transposable genetic elements. Integration is thought to occur essentially at random within the target cell genome. However, by modifying the long-terminal repeats it is possible to control the integration of a retroviral genome.

Transcription, RNA splicing and translation of the integrated viral DNA is mediated by host cell proteins. Variously spliced transcripts are generated. In the case of the human retroviruses HIV-1/2 and HTLV-I/II viral proteins are also used to regulate gene expression. The interplay between cellular and viral factors is important in the control of virus latency and the temporal sequence in which viral genes are expressed.

Retroviruses can be transmitted horizontally and vertically. Efficient infectious transmission of retroviruses requires the expression on the target cell of receptors which specifically recognize the viral envelope proteins, although viruses may use receptor-independent, nonspecific routes of entry at low efficiency. In addition, the target cell type must be able to support all stages of the replication cycle after virus has bound and penetrated. Vertical transmission occurs when the viral genome becomes integrated in the germ line of the host. The provirus will then be passed from generation to generation as though it were a cellular gene. Hence endogenous proviruses become established which frequently lie latent, but which can become activated when the host is exposed to appropriate agents.

Sequence analysis showed that a $JSRV_{21}$, isolated as described below, possesses the hallmarks of integrated retroviral proviruses, such as the presence of a CA-TG dinucleotide pair present at the termini of the upstream and downstream viral LTRs, the loss of 2 nucleotides (nt) from the termini of the LTRs during integration,

and an apparent duplication of 6 nt of cellular flanking sequences (TGTGTC (SEQ ID NO:___)) at the integration site. The flanking cellular sequences in the $JSRV_{21}$ clone were 393 and 1,006 bp long and did not align with known cellular sequences (including proto-oncogenes).

In the early steps of infection, retroviruses deliver their nucleoprotein core into the cytoplasm of the target cell. Here, reverse transcription of the viral genome takes place while the core matures into a preintegration complex. The complex must reach the nucleus to achieve integration of the viral DNA into the host cell chromosomes. For simple retroviruses (oncoretroviruses), this step requires the dissolution of the nuclear membrane at mitotic prophase, most likely because the bulky size of the preintegration complex prevents its passive diffusion through the nuclear pores because there are no nuclear localization signals to facilitate active transport into the nucleus.

A $JSRV_{21}$ provirus is 7,834 bp long, and the viral genome (R to R) is 7,455 nt. $JSRV_{21}$ shows the characteristic genomic organization of type D and type B retroviruses, with *pro* in a different open reading frame from *pol* (FIG. 2a). $JSRV_{21}$ showed homology to JSRV-SA. The homology was 90% in the LTRs (89% in U3), 91% in *gag*, 96% in *pol*, and 91% in *env*. $JSRV_{21}$ is 7 bp shorter than JSRV-SA and in particular has a 5-bp deletion in U3 with respect to JSRV-SA. One difference between the coding regions of JSRV-SA and $JSRV_{21}$ was in the *pro* region: the *pro* open reading frame in $JSRV_{21}$ starts 53 nt downstream from the putative *pro* start in JSRV-SA; in particular, there are two stop codons in $JSRV_{21}$ at positions 1919 and 1931 that are not present in JSRV-SA. Thus, for $JSRV_{21}$, the translational frameshift that presumably occurs during synthesis of the gag-pro-pol polyprotein precursor must occur downstream of the stop codon at nt 1932. It is interesting that the gag protein sequences for these two viruses have 100% identity in the region shown, so that the differences in the *pro* sequences did not affect the overlapping *gag* gene product. The *orf-x* open reading frame first identified in JSRV-SA was conserved in $pJSRV_{21}$, suggesting that it plays a functional role. The nucleic acid sequence of $JSRV_{21}$ has accession number AF105220, which is hereby incorporated by reference in its entirety.

Animal retroviruses have provided great insights into steps in oncogenesis for both animal and human cancers. However, with the notable exception of murine mammary tumor virus, most oncogenic retroviruses typically induce tumors of the hematopoietic system. JSRV is unique among retroviruses in transforming lung epithelial cells (type II pneumocytes and Clara cells). The strong resemblance of human BAC and ovine SPA suggests that studies of JSRV oncogenesis provide new insights into the development of human BAC. SPA is a naturally occurring disease of an outbred animal species and therefore may be a particularly useful animal model for the human disease.

Interestingly, two other JSRV-related retroviruses of small ruminants, enzootic nasal tumor virus of sheep and goats, are associated with tumors of the ethmoid turbinates that arise from secretory epithelial cells. Thus, small-ruminant type D retroviruses as described herein offer novel insight into oncogenic mechanisms in secretory epithelial cells.

Other oncogenic retroviruses exert their pathogenic effects by carrying transforming genes (oncogenes) or by insertionally activating cellular proto-oncogenes. It is noteworthy that JSRV induces lung cancer in sheep quite rapidly: 4 months in these experiments and as quickly as 3 to 4 weeks in previous experiments with uncloned virus. Moreover, the pattern of the tumor cells was more consistent with multifocal disease. A histology of tissue showed the induction of SPA in $JSRV_{21}$-infected lambs. Lung tumor tissues from $JSRV_{21}$-infected lambs were fixed in neutral 10% formalin, embedded in paraffin, and sectioned by routine procedures. Hematoxylin and eosin-stains were used in the lung tumor sections. A low-magnification micrograph showed many neoplastic foci in the microscopic field. A high-magnification micrograph (magnification, ×372; bar, 40 μm) of a neoplastic nodule with a clear papillary pattern showed myxoid tissue containing cells with elongated or round nuclei in the interstitium of the neoplastic tissue. A papillary proliferation was also seen occluding the lumen of a bronchiolus. Immunohistochemistry for JSRV CA antigen developed with an avidin-biotin peroxidase complex kit (ABC; Vector Laboratories) and a Carazzi's hematoxylin alone as counterstain showed a neoplastic focus indicative of JSRV CA antigen. No

staining was present in the cells infiltrating the tumor or in adjacent normal cells. A lung section from an uninoculated lamb was tested for JSRV CA antigen under conditions similar to those described above; there were no antigen-positive cells. By analogy to other retrovirus complexes that induce disease rapidly, it initially seemed possible that a defective acute transforming retrovirus carrying a viral oncogene was the cause of the SPA tumors. However, the cloned $JSRV_{21}$ can induce disease within the same time frame as field isolates. Thus, the oncogenic potential for SPA is contained within the $JSRV_{21}$ sequences, even though no obvious oncogenes with homology to cellular proto-oncogenes are present. On the other hand, insertional activation of proto-oncogenes is typically associated with multiple rounds of infection, high viral loads, and long incubation periods. Previous results suggest that JSRV oncogenesis may not fit this paradigm either. In animals with spontaneous or experimentally induced SPA, the only cells in which JSRV protein can be detected are the tumor cells themselves. In particular, in these animals, normal lung epithelial cells do not show detectable viral antigen. Also, in experimentally infected animals, viral DNA in circulating blood cells can be detected only by extremely sensitive nested PCR techniques, and there is no evidence for viral expression.

As described herein the JSRV genome carries an alternate open reading frame (orf-x) overlapping pol. This reading frame shows no homologies to any other known gene (viral or cellular). The fact that orf-x is conserved as an open reading frame for both the South African and British isolates of JSRV (JSRV-SA and $JSRV_{21}$) strongly suggests that it plays a role in viral replication, oncogenesis, or both.

As used herein, the term "isolated" means altered "by the hand of man" from its natural state; *i.e.*, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living animal in its natural state is not "isolated", but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. As part of or following isolation, a polynucleotide can be joined to other polynucleotides, such as for example DNAs, for mutagenesis studies, to form fusion proteins, and for propagation or expression of the polynucleotide in a host. The isolated polynucleotides, alone or joined

to other polynucleotides, such as vectors, can be introduced into host cells, in culture or in whole organisms. Such polynucleotides, when introduced into host cells in culture or in whole organisms, still would be isolated, as the term is used herein, because they would not be in their naturally occurring form or environment. Similarly, the polynucleotides and polypeptides may occur in a composition, such as a media formulation (solutions for introduction of polynucleotides or polypeptides, for example, into cells or compositions or solutions for chemical or enzymatic reactions).

Polynucleotide or nucleic acid sequence refers to a polymeric form of nucleotides. In some instances a polynucleotide refers to a sequence that is not immediately contiguous with either of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA) independent of other sequences. The polynucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. In addition, the polynucleotide sequence involved in producing a polypeptide chain can include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons) depending upon the source of the polynucleotide sequence.

The term polynucleotide(s) generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions.

In addition, a polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be

from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide.

In addition, the polynucleotides or nucleic acid sequences may contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

Nucleic acid sequences can be created which encode a fusion protein and can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a coding sequence is "operably linked" to another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences ultimately process to produce the desired protein. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, ribosomal binding sites, transcription terminators, a start codon (*i.e.*, ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of the correct reading frame of that gene to permit proper translation of the mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the of the polynucleotide sequence. For example, the LTR regions of the JSRV of the present invention have a degree of specificity for pulmonary (*e.g.*, lung) epithelial cells. Both constitutive and inducible promoters, are included in the invention (see *e.g.*, Bitter *et al.*, Methods in Enzymology 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as *pL* of bacteriophage, *plac*, *ptrp*, *ptac* (*ptrp-lac* hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

As described more fully below, a heterologous nucleic acid sequence or a fragment or portion of the JSRV genome of the invention may be inserted into a recombinant expression vector. A recombinant expression vector generally refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of a nucleic acid sequences. For example, a recombinant expression vector of the invention includes the JSRV genomic sequence, or a fragment thereof, containing a heterologous polynucleotide sequence, or a fragment of the JSRV sequence (*e.g.*, and LTR sequence of JSRV) linked to a sequence encoding a polynucleotide of interest in order to provide tissue specific regulation of the sequence of interest. The expression vector typically contains an origin of replication, one or more regulatory sequences, and can also contain specific genes which allow phenotypic selection of a transformed cell. Vectors suitable for use in the invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg, *et al.*, Gene 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, J. Biol. Chem. 263:3521, 1988), baculovirus-derived vectors for expression in insect cells, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV. The nucleic acid sequences of the invention can also include a localization sequence to

direct the indicator to particular cellular sites by fusion to appropriate organellar targeting signals or localized host proteins. For example, a polynucleotide encoding a localization sequence, or signal sequence, can be used as a repressor and thus can be ligated or fused at the 5' terminus of a polynucleotide encoding a polypeptide of the invention such that the localization or signal peptide is located at the amino terminal end of a resulting polynucleotide/polypeptide. The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art. (See, for example, Sambrook *et al.*, Molecular Cloning --A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989, and Current Protocols in Molecular Biology, M. Ausubel *et al.*, eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., most recent Supplement)). These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. (See also, Maniatis, *et al.*, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., 1989).

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel, *et al.*, Greene Publish. Assoc. & Wiley Interscience, Ch. 13, 1988; Grant, *et al.*, "Expression and Secretion Vectors for Yeast," in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp.516-544, 1987; Glover, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3, 1986; and Bitter, "Heterologous Gene Expression in Yeast," Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684, 1987; and The Molecular Biology of the Yeast Saccharomyces, Eds. Strathern *et al.*, Cold Spring Harbor Press, Vols. I and II, 1982. A constitutive yeast promoter such as *ADH* or *LEU2* or an inducible promoter such as *GAL* may be used ("Cloning in Yeast," Ch. 3, R. Rothstein In: DNA Cloning Vol.11, A Practical Approach, Ed. DM Glover, IRL Press, Wash., D.C., 1986). Alternatively, vectors may be used which promote integration of foreign DNA sequences into the yeast chromosome.

An alternative expression system which could be used to express a polypeptide (*e.g.*, a polypeptide of JSRV) is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign or

mutated polynucleotide sequences. The virus grows in *Spodoptera frugiperda* cells. The sequence encoding a protein of the invention may be cloned into non-essential regions (for example, the polyhedrin gene) of the virus and placed under control of an *AcNPV* promoter (for example the polyhedrin promoter). Successful insertion of the sequences coding for a protein of the invention will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *S. frugiperda* cells in which the inserted gene is expressed, see Smith, *et al.*, J. Viol. 46:584, 1983; Smith, U.S. Patent No. 4,215,051.

The vectors, including a recombinant JSRV sequence, of the invention can be used to transform a host cell. By transform or transformation is meant a permanent or transient genetic change induced in a cell following incorporation of new DNA (*i.e.*, DNA exogenous to the cell). Where the cell is a mammalian cell, a permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell.

A transformed cell or host cell generally refers to a cell (*e.g.*, prokaryotic or eukaryotic) into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding a polypeptide of interest or a fragment thereof.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method by procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation.

When the host is a eukaryote, methods of transfection or transformation with DNA include calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid or vector of the invention contained in liposomes, or virus vectors, as well as others known in the art, may be used. Eukaryotic cells can also be cotransfected with DNA sequences of the invention and a second foreign DNA molecule encoding a selectable marker, such as the herpes simplex

thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus containing the JSRV sequence or a fragment thereof, to transiently infect or transform eukaryotic cells and express or replicate the sequence. (Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982). Typically, a eukaryotic host will be utilized as the host cell. The eukaryotic cell may be a yeast cell (*e.g.*, *Saccharomyces cerevisiae*), an insect cell (*e.g.*, *Drosophila sp.*) or may be a mammalian cell such as equine, bovine, canine, feline, orvine, and include primate and human cells. Typically the cell will be a mammalian cell derived from the pulmonary system, including the lung, trachea or bronchia (*e.g.*, tracheal or bronchial epithelial cell lines and primary cells).

Eukaryotic systems, and mammalian expression systems, allow for post-translational modifications of expressed mammalian proteins to occur. Eukaryotic cells which possess the cellular machinery for processing of the primary transcript, glycosylation, phosphorylation, and, advantageously secretion of the gene product should be used. Such host cell lines may include, but are not limited to, MLE-15, mtCC1-2, CHO, VERO, BHK, HeLa, COS, MDCK, Jurkat, HEK-293, and WI38.

Mammalian cell systems which utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, a polynucleotide encoding a JSRV polypeptide may be ligated to an adenovirus transcription/ translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric sequence may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the polypeptide of interest or a fragment thereof in infected hosts (*e.g.*, see Logan & Shenk, Proc. Natl. Acad. Sci. USA, 81:3655-3659, 1984). Alternatively, the vaccinia virus 7.5K promoter may be used. (*e.g.*, see, Mackett, *et al.*, Proc. Natl. Acad. Sci. USA, 79:7415-7419, 1982; Mackett, *et al.*, J. Virol. 49:857-864, 1984; Panicali, *et al.*, Proc. Natl. Acad. Sci. USA 79:4927-4931, 1982). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver, *et al.*, Mol. Cell. Biol. 1:486, 1981). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the

host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the *neo* gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of a heterologous polynucleotide sequence encoding a therapeutic or non-therapeutic protein in host cells (Cone & Mulligan, Proc. Natl. Acad. Sci. USA, 81:6349-6353, 1984). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionine IIA promoter and heat shock promoters.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with a JSRV polynucleotide or a polynucleotide encoding a JSRV polypeptide controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. The selectable marker in the recombinant vector confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler, *et al.*, Cell, 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci. USA, 48:2026, 1962), and adenine phosphoribosyltransferase (Lowy, *et al.*, Cell, 22:817, 1980) genes can be employed in *tk-*, *hgprt-* or *aprt-* cells respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, *et al.*, Proc. Natl. Acad. Sci. USA, 77:3567, 1980; O'Hare, *et al.*, Proc. Natl. Acad. Sci. USA, 8:1527, 1981); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci. USA, 78:2072, 1981; *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, J. Mol. Biol. 150:1, 1981); and *hygro*, which confers resistance to hygromycin (Santerre, *et al.*, Gene 30:147, 1984) genes. Recently, additional selectable genes have been described, namely *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in

place of histidine (Hartman & Mulligan, Proc. Natl. Acad. Sci. USA 85:8047, 1988); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, ed., 1987).

The term "primer" as used herein refers to an oligonucleotide, whether natural or synthetic, which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated or possible. Synthesis of a primer extension product which is complementary to a nucleic acid strand is initiated in the presence of nucleoside triphosphates and a polymerase in an appropriate buffer at a suitable temperature. For instance, if a nucleic acid sequence is inferred from a protein sequence, a primer generated to synthesize nucleic acid sequence encoding the protein sequence is actually a collection of primer oligonucleotides containing sequences representing all possible codon variations based on the degeneracy of the genetic code. One or more of the primers in this collection will be homologous with the end of the target sequence. Likewise, if a "conserved" region shows significant levels of polymorphism in a population, mixtures of primers can be prepared that will amplify adjacent sequences.

A polypeptide or protein refers to a polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being typical. A polypeptide of the invention includes an amino acid sequence encoded by a JSRV comprised of L- or D- amino acids and include modified sequences such as glycoproteins. Accordingly, the polypeptides of the invention are intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically synthesized. Polypeptide or protein fragments are also encompassed by the invention. Fragments can have the same or substantially the same amino acid sequence as the naturally occurring protein. A polypeptide or peptide having substantially the same sequence means that an amino acid sequence is largely, but not entirely, the same, but retains a functional activity of the sequence to which it is related. In general polypeptides of the invention include peptides, or full length protein, that contains substitutions, deletions, or insertions into the protein backbone, that would still have an approximately 70%-90% homology to the original protein over the

corresponding portion. A yet greater degree of departure from homology is allowed if like-amino acids, *i.e.* conservative amino acid substitutions, do not count as a change in the sequence

A polypeptide may be substantially related but for a conservative variation, such polypeptides being encompassed by the invention. A conservative variation denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. Other illustrative examples of conservative substitutions include the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine, glutamine, or glutamate; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; valine to isoleucine to leucine. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

Modifications and substitutions are not limited to replacement of amino acids. For a variety of purposes, such as increased stability, solubility, or configuration concerns, one skilled in the art will recognize the need to introduce, (by deletion, replacement, or addition) other modifications. Examples of such other modifications include incorporation of rare amino acids, dextra-amino acids, glycosylation sites, cytosine for specific disulfide bridge formation. The modified peptides can be chemically synthesized, or the isolated gene can be site-directed mutagenized, or a synthetic gene can be synthesized and expressed in bacteria, yeast, baculovirus, tissue culture and so on.

In one embodiment, the invention provides an isolated polynucleotide sequence corresponding to the isolated genome of JSRV. Polynucleotide sequences of the

invention include DNA, cDNA and RNA sequences. It is understood that all polynucleotides encoding all or a portion of a JSRV genome are included herein. Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, a JSRV polynucleotide of the invention includes the JSRV sequence having accession number AF105220. In addition, a polynucleotide of the invention includes a fragment of the sequence having accession number AF105220 as well as sequences subjected to site-directed mutagenesis. The polynucleotides of the invention include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention so long as a protein encoded by a JSRV genome is functionally unchanged.

In addition, the present invention provides polynucleotide sequence encoding a recombinant JSRV vector of the present invention. The JSRV polynucleotide sequence can be incorporated into various viral particles.

The present invention also provides means for isolating and identifying related viral sequences or polynucleotide from other organisms, including humans. For example, one may probe a gene library with a natural or artificially designed probe using art recognized procedures (see, for example: Current Protocols in Molecular Biology, Ausubel F.M. *et al.* (Eds.) Green Publishing Company Assoc. and John Wiley Interscience, New York, 1989, 1992). It is appreciated by one skilled in the art that probes can be designed based on the degeneracy of the genetic code to a sequences corresponding to a polypeptide or polynucleotide of the invention.

In addition, sequencing algorithms can be used to measure homology or identity between known and unknown sequences. Such methods and algorithms are useful in identifying corresponding sequences present in other organisms. Homology or identity is often measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences

that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequence for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol 48:443 (1970), by the search for similarity method of person & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

One example of a useful algorithm is BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, Nuc. Acids Res. 25:3389-3402 (1977) and Altschul *et al.*, J. Mol. Biol. 215:403-410 (1990), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with

a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al., supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) or 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectations (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873 (1993)). One measure of similarity provided by BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a references sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

Other algorithms for determining homology or identity include, for example, in addition to a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information), ALIGN, AMAS (Analysis of Multiply Aligned Sequences), AMPS (Protein Multiple Sequence Alignment), ASSET (Aligned Segment Statistical Evaluation Tool), BANDS, BESTSCOR, BIOSCAN (Biological

Sequence Comparative Analysis Node), BLIMPS (BLocks IMProved Searcher), FASTA, Intervals & Points, BMB, CLUSTAL V, CLUSTAL W, CONSENSUS, LCONSENSUS, WCONSENSUS, Smith-Waterman algorithm, DARWIN, Las Vegas algorithm, FNAT (Forced Nucleotide Alignment Tool), Framealign, Framesearch, DYNAMIC, FILTER, FSAP (Fristensky Sequence Analysis Package), GAP (Global Alignment Program), GENAL, GIBBS, GenQuest, ISSC (Sensitive Sequence Comparison), LALIGN (Local Sequence Alignment), LCP (Local Content Program), MACAW (Multiple Alignment Construction & Analysis Workbench), MAP (Multiple Alignment Program), MBLKP, MBLKN, PIMA (Pattern-Induced Multi-sequence Alignment), SAGA (Sequence Alignment by Genetic Algorithm) and WHAT-IF. Such alignment programs can also be used to screen genome databases to identify polynucleotide sequences having substantially identical sequences. A number of genome databases are available, for example, a substantial portion of the human genome is available as part of the Human Genome Sequencing Project (J. Roach, http://weber.u.Washington.edu/~roach/human_ genome_ progress2.html) (Gibbs, 1995). At least twenty-one other genomes have already been sequenced, including, for example, *M. genitalium* (Fraser *et al.*, 1995), M. jannaschii (Bult *et al.*, 1996), *H. influenzae* (Fleischmann *et al.*, 1995), *E. coli* (Blattner *et al.*, 1997), and yeast (S. cerevisiae) (Mewes *et al.*, 1997), and *D. melanogaster* (Adams *et al.*, 2000). Significant progress has also been made in sequencing the genomes of model organism, such as mouse, *C. elegans*, *Arabadopsis sp.* and *D. melanogaster*. Several databases containing genomic information annotated with some functional information are maintained by different organization, and are accessible via the internet, for example, http://wwwtigr.org/tdb; http://www.genetics.wisc.edu; http://genome-www.stanford.edu/~ball; http://hiv-web.lanl.gov; http://www.ncbi.nlm.nih.gov; http://www.ebi.ac.uk; http://Pasteur.fr/other/biology; and http://www.genome.wi.mit.edu.

A "substantially pure polypeptide" is typically pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, the polypeptide of interest. A substantially pure polypeptide includes substantially pure viral particles and may be obtained, for example, by extraction from a natural source (*e.g.*, lung tissue or lung carcinoma tissue); by expression of a recombinant nucleic acid encoding or JSRV

genome; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

In addition to polypeptides of the invention, specifically disclosed herein is a DNA sequence for a jaagsiekte sheep retrovirus. DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization or computer-based techniques which are well known in the art. These include, but are not limited to: 1) hybridization of genomic libraries with probes to detect homologous nucleotide sequences; 2) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features; 3) polymerase chain reaction (PCR) on genomic DNA using primers capable of annealing to the DNA sequence of interest; and 4) computer searches of sequence databases for similar sequences as described above.

The polynucleotide of the invention (*e.g.*, the JSRV polynucleotide sequence) includes complementary polynucleotide sequences, as well as splice variants thereof. When the sequence is RNA, the deoxyribonucleotides A, G, C, and T are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments (portions) of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA that encodes a polypeptide sequence of the invention. "Selective hybridization" as used herein refers to hybridization under moderately stringent or highly stringent physiological conditions (See, for example, the techniques described in Maniatis *et al.*, 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., incorporated herein by reference), which distinguishes related from unrelated nucleotide sequences.

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization

conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42 °C (moderate stringency conditions); and 0.1 x SSC at about 68 °C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

Oligonucleotides encompassed by the present invention are also useful as primers for nucleic acid amplification reactions. In general, the primers used according to the method of the invention embrace oligonucleotides of sufficient length and appropriate sequence which provides specific initiation of polymerization of a significant number of nucleic acid molecules containing the target nucleic acid under the conditions of stringency for the reaction utilizing the primers. In this manner, it is possible to selectively amplify the specific target nucleic acid sequence containing the nucleic acid of interest.

Amplified products may be detected by Southern blot analysis, without using radioactive probes. In such a process, for example, a small sample of DNA containing a very low level of a nucleotide sequence is amplified and analyzed via a Southern blotting technique known to those of skill in the art. The use of non-radioactive probes or labels is facilitated by the high level of the amplified signal.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. For example, it is envisioned that such probes can be used to identify other related or family members of the jaagsiekte sheep retrovirus. In accomplishing this, alignment algorithms (as described above) can be used to screen genome databases. Alternatively, oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that

short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of DNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al.*, Nucl. Acid Res., 9:879, 1981).

When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is use of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned.

In the invention, a JSRV polynucleotide sequence may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of a JSRV genetic sequence. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include those described above.

Methods, which are well known to those skilled in the art, can be used to construct expression vectors containing a JSRV polynucleotide or JSRV coding sequence and appropriate transcriptional/translational control signals. These methods

include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic techniques. (See, for example, the techniques described in Maniatis *et al.*, 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.).

The genetic construct can be designed to provide additional benefits, such as, for example addition of C-terminal or N-terminal amino acid residues that would facilitate purification by trapping on columns or by use of antibodies. All those methodologies are cumulative. The choice as to the method of producing a particular construct can easily be made by one skilled in the art based on practical considerations: size of the desired peptide, availability and cost of starting materials, *etc.* All the technologies involved are well established and well known in the art. See, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, Volumes 1 and 2 (1987), with supplements, and Maniatis *et al.*, Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory (1989). Yet other technical references are known and easily accessible to one skilled in the art.

In another embodiment, the invention provides antibodies that bind to a JSRV viral particle (*e.g.*, env polypeptide) of the invention. Such antibodies are useful for research and diagnostics in the study and treatment of transmission of JSRV as well as related diseases (*e.g.*, lung carcinomas), and associated pathologies in general.

Such antibodies may be administered alone or contained in a pharmaceutical composition comprising antibodies against a JSRV polypeptide (*e.g.*, env antigen) and other reagents effective as modulators of the interaction of an JSRV env polypeptide with its ligand both *in vitro* and *in vivo*.

The term "epitope", as used herein, refers to an antigenic determinant on an antigen, such as an env polypeptide, to which the paratope of an antibody, such as an antibody that binds to a JSRV encoded polypeptide (*e.g.*, an env polypeptide) of the invention. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics.

Antibodies which bind to a polypeptide of the invention can be prepared using an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or a peptide used to immunize an animal can be derived from translated cDNA or chemical synthesis which can be conjugated to a carrier protein, if desired. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (*e.g.*, a mouse, a rat, or a rabbit).

If desired, polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (See for example, Coligan, *et al.*, Unit 9, Current Protocols in Immunology, Wiley Interscience, 1991, incorporated by reference).

It is also possible to use the anti-idiotype technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody.

Antibodies of the invention include polyclonal antibodies, monoclonal antibodies, and fragments of polyclonal and monoclonal antibodies.

The preparation of polyclonal antibodies is well-known to those skilled in the art. See, for example, Green *et al.*, Production of Polyclonal Antisera, in Immunochemical Protocols (Manson, ed.), pages 1-5 (Humana Press 1992); Coligan *et al.*, Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters, in Current Protocols in Immunology, section 2.4.1 (1992), which are hereby incorporated by reference.

The preparation of monoclonal antibodies likewise is conventional. See, for example, Kohler & Milstein, Nature, 256:495 (1975); Coligan *et al.*, sections 2.5.1-2.6.7; and Harlow *et al.*, Antibodies: A Laboratory Manual, page 726 (Cold Spring Harbor Pub. 1988), which are hereby incorporated by reference. Briefly, monoclonal antibodies

can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, *e.g.*, Coligan *et al.*, sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes *et al.*, Purification of Immunoglobulin G (IgG), in Methods in Molecular Biology, Vol. 10, pages 79-104 (Humana Press 1992). Methods of *in vitro* and *in vivo* multiplication of monoclonal antibodies is well-known to those skilled in the art. Multiplication *in vitro* may be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally replenished by a mammalian serum such as fetal calf serum or trace elements and growth-sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* may be carried out by injecting cell clones into mammals histocompatible with the parent cells, *e.g.*, osyngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane tetramethylpentadecane prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

Therapeutic applications for antibodies disclosed herein are also part of the present invention. For example, antibodies of the present invention may also be derived from subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in Goldenberg *et al.*, International Patent Publication WO 91/11465 (1991) and Losman *et al.*, Int. J. Cancer, 46:310 (1990), which are hereby incorporated by reference.

Alternatively, an anti-JSRV antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, Proc. Nat'l Acad. Sci. USA, 86:3833 (1989), which is hereby incorporated in its entirety by reference. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, Nature, 321:522 (1986); Riechmann *et al.*, Nature, 332:323 (1988); Verhoeyen *et al.*, Science, 239:1534 (1988); Carter *et al.*, Proc. Nat'l Acad. Sci. USA, 89:4285 (1992); Sandhu, Crit. Rev. Biotech., 12:437 (1992); and Singer *et al.*, J. Immunol., 150:2844 (1993), which are hereby incorporated by reference.

Antibodies of the invention also may be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas *et al.*, Methods: A Companion to Methods in Enzymology, Vol. 2, page 119 (1991); Winter *et al.*, Ann. Rev. Immunol. 12:433 (1994), which are hereby incorporated by reference. Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from STRATAGENE Cloning Systems (La Jolla, CA).

In addition, antibodies of the present invention may be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green *et al.*, Nature Genet., 7:13 (1994); Lonberg *et al.*, Nature, 368:856 (1994); and Taylor *et al.*, Int. Immunol., 6:579 (1994), which are hereby incorporated by reference.

Antibody fragments of the invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patents No. 4,036,945 and No. 4,331,647, and references contained therein. These patents are hereby incorporated in their entireties by reference. See also Nisonhoff *et al.*, Arch. Biochem. Biophys,. 89:230 (1960); Porter, Biochem. J., 73:119 (1959); Edelman *et al.*, Methods in Enzymology, Vol. 1, page 422 (Academic Press 1967); and Coligan *et al.* at sections 2.8.1-2.8.10 and 2.10.1-2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of V_H and V_L chains. This association may be noncovalent, as described in Inbar *et al.*, Proc. Nat'l Acad. Sci. USA, 69:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. See, *e.g.*, Sandhu, *supra*. Preferably, the F_v fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow *et al.*, Methods: A Companion to Methods in Enzymology, Vol. 2, page 97 (1991); Bird *et al.*, Science,

242:423 (1988); Ladner *et al.*, U.S. patent No. 4,946,778; Pack *et al.*, Bio/Technology, 11:1271 (1993); and Sandhu, *supra.*

Another form of an antibody fragment is a peptide coding for a single complementarity- determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick *et al.*, Methods: A Companion to Methods in Enzymology, Vol. 2, page 106 (1991).

In one embodiment, the invention provides a method for modulating (*e.g.*, inhibiting) the interaction of a JSRV env polypeptide with its ligand (either *in vitro* or *in vivo*) by administering to a cell or subject an effective amount of a composition which contains an env polypeptide, or biologically functional fragment thereof or an agent (e.g, an antibody, ribozyme, antisense molecule, or double-stranded interfering RNA molecules) that interacts with or inhibits expression of a JSRV polypeptide.

As used herein, an "effective amount" of a composition containing a JSRV - modulating agent is defined as that amount that is effective in modulating normal transduction or interaction of a JSRV in a subject or cell.

In another embodiment, the present invention provides a method for modulating expression of a JSRV polypeptide as well as methods for screening for agents which modulate JSRV gene expression. In this embodiment, a cell or subject is contacted with an agent suspected or known to have JSRV expression modulating activity. The change in JSRV gene expression is then measured as compared to a control or standard sample. The control or standard sample can be the baseline expression of the cell or subject prior to contact with the agent. An agent which modulates JSRV gene expression may be a polynucleotide, for example, the polynucleotide may be an antisense, a triplex agent, a ribozyme, or a double-stranded interfering RNA. For example, an antisense molecule may be directed to the structural gene region or to the promoter region (*e.g.*, the LTR region) of JSRV. The agent may be an agonist, antagonist, peptide, peptidomimetic, antibody, or chemical.

It is envisioned that the invention can be used to treat pathologies associated with JSRV, inlcuding cell proliferative disorders, such as lung cancer. Therefore, the present invention encompasses methods for ameliorating a disorder associated with JSRV, including treating a subject having the disorder, at the site of the disorder, with an agent which modulates JSRV expression or its interaction with its ligand resulting in infection. Generally, the terms "treating", "treatment" and the like are used herein to mean affecting a subject, tissue or cell to obtain a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or sign or symptom thereof, and/or may be therapeutic in terms of a partial or complete cure for an infection or disease and/or adverse effect attributable to the infection or disease. "Treating" as used herein covers any treatment of, or prevention of a disease in an invertebrate, a vertebrate, a mammal, particularly a human, and includes: (a) preventing the disorder from occurring in a subject that may be predisposed to the disorder, but has not yet been diagnosed as having it; (b) inhibiting the disorder, *i.e.*, arresting its development; or (c) relieving or ameliorating the disorder, *i.e.*, cause regression of the disorder.

The invention includes various pharmaceutical compositions useful for treating or ameliorating symptoms attributable to a JSRV-associated disorder. The pharmaceutical compositions according to one embodiment of the invention are prepared by bringing an antibody against a JSRV polypeptide (*e.g.*, an env polypeptide), a drug, chemical or combination of chemicals or a JSRV-modulating agent into a form suitable for administration to a subject using carriers, excipients and additives or auxiliaries. Frequently used carriers or auxiliaries include magnesium carbonate, titanium dioxide, lactose, mannitol and other sugars, talc, milk protein, gelatin, starch, vitamins, cellulose and its derivatives, animal and vegetable oils, polyethylene glycols and solvents, such as sterile water, alcohols, glycerol and polyhydric alcohols. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described, for instance, in Remington's Pharmaceutical Sciences, 15th ed. Easton: Mack Publishing Co., 1405-1412, 1461-1487 (1975) and The National Formulary XIV., 14th ed. Washington: American

Pharmaceutical Association (1975), the contents of which are hereby incorporated by reference. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art. See Goodman and Gilman's The Pharmacological Basis for Therapeutics (7th ed.).

The pharmaceutical compositions are preferably prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories. For treatment of a subject, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the subject, different daily doses are necessary. Under certain circumstances, however, higher or lower daily doses may be appropriate. The administration of the daily dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

The pharmaceutical compositions according to the invention may be administered locally or systemically in a therapeutically effective dose. Amounts effective for this use will, of course, depend on the severity of the disease and the weight and general state of the subject. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, *e.g.*, in Langer, Science, 249:1527, (1990); Gilman *et al.* (eds.) (1990), each of which is herein incorporated by reference.

"Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Preferably a "subject" refers to a mammal, most preferably a human, but may be any organism, including sheep or other domesticated animals.

An anti-JSRV antibody can be administered parenterally, enterically, by injection, rapid infusion, nasopharyngeal absorption, dermal absorption, rectally and orally. Pharmaceutically acceptable carrier preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers for

occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners and elixirs containing inert diluents commonly used in the art, such as purified water.

In another embodiment, the invention provides a method for identifying an agent which interacts with or modulates expression or activity of JSRV including incubating components comprising an agent and a cell or culture containing a replication competent JSRV, or a recombinant cell expressing a JSRV viral particle, under conditions sufficient to allow the agent to interact and determining the affect of the agent on the expression, activity, or infectivity of the gene, polypeptide, or JSRV, respectively. The term "affect", as used herein, encompasses any means by which gene expression or protein activity can be modulated, and includes measuring the interaction of the agent with the JSRV by physical means including, for example, fluorescence detection of the binding of a ligand to the receptor. Such agents can include, for example, polypeptides, peptidomimetics, chemical compounds, small molecules and biologic agents as described below.

Incubating includes conditions which allow contact between the test agent and a JSRV polypeptide, a cell expressing JSRV or a JSRV nucleic acid sequence. Contacting includes in solution and in solid phase. The test agent may optionally be a combinatorial library for screening a plurality of agents. Agents identified in the method of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki, *et al.*, Bio/Technology, 3:1008-1012, 1985), oligonucleotide ligation assays (OLAs) (Landegren, *et al.*, Science, 241:1077, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landegren, *et al.*, Science, 242:229-237, 1988).

Thus, the method of the invention includes combinatorial chemistry methods for identifying chemical agents that bind to or affect JSRV gene expression or JSRV infectivity.

Areas of investigation are the development of therapeutic treatments. The screening identifies agents that provide modulation of JSRV gene function and/or infectivity in targeted organisms. Of particular interest are screening assays for agents that have a low toxicity or a reduced number of side effects for mammals, such as sheep and humans.

The term "agent" as used herein describes any molecule, *e.g.* protein or pharmaceutical, with the capability of altering or mimicking the physiological function or expression of a JSRV gene or a JSRV polypeptide. Generally, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

In addition, cells or organisms which have a mutations in a JSRV polypeptide or polynucleotide sequence may be used as models to screen for agents which modulate disorders associated with the mutation.

In a further embodiment, the invention provides a method of detecting a JSRV, a JSRV polypeptide or a JSRV polynucleotide or diagnosing a JSRV-related disorder (*e.g.*, cancer) in a subject including contacting a cell component suspected of containing a JSRV polypeptide or a JSRV polynucleotide with a reagent which binds to the polypeptide or polynucleotide (herein after cell component). The cell component can be or contain a nucleic acid, such as DNA or RNA, or a protein. When the component is nucleic acid, the reagent is a nucleic acid probe or PCR primer. When the cell component is protein, the reagent is an antibody probe. The probes are detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other labels suitable for binding to an antibody or nucleic acid probe, or will be able to ascertain such, using routine experimentation. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention

include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds. In addition, the antibodies, polypeptides and polynucleotide sequences of the invention can be used to diagnosis a JSRV-related disorder.

A monoclonal antibody of the invention, directed toward a JSRV polypeptide (*e.g.*, an env polypeptide) is useful for the *in vivo* and *in vitro* detection of antigen. The detectably labeled monoclonal antibody is given in a dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled monoclonal antibody is administered in sufficient quantity to enable detection of a JSRV, or a JSRV polypeptide antigen for which the monoclonal antibodies are specific.

The concentration of a detectably labeled monoclonal antibody administered to a subject should be sufficient such that the binding to those cells, body fluid, or tissue having a JSRV polypeptide that is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 key range, which may be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA)

and similar molecules. Typical examples of metallic ions which can be bound to the monoclonal antibodies of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl.

The monoclonal antibodies of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

In another embodiment, nucleic acid probes can be used to identify a JSRV polynucleotide from a specimen obtained from a subject. Examples of specimens from which nucleic acid sequence encoding a retrovirus of the invention (e.g., JSRV) can be derived include insect, human, primate, swine, porcine, feline, canine, equine, murine, cervine, caprine, lupine, leporidine, opine and bovine species.

Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al.*, Nucl. Acid Res. 9:879, 1981).

In an embodiment of the invention, purified nucleic acid fragments containing intervening sequences or oligonucleotide sequences of 10-50 base pairs are radioactively

labeled. The labeled preparations are used to probe nucleic acids from a specimen by the Southern hybridization technique. Nucleotide fragments from a specimen, before or after amplification, are separated into fragments of different molecular masses by gel electrophoresis and transferred to filters that bind nucleic acid. After exposure to the labeled probe, which will hybridize to nucleotide fragments containing target nucleic acid sequences, binding of the radioactive probe to target nucleic acid fragments is identified by autoradiography (see Genetic Engineering, 1, ed. Robert Williamson, Academic Press, (1981), 72-81). Alternatively, nucleic acid from the specimen can be bound directly to filters to which the radioactive probe selectively attaches by binding nucleic acids having the sequence of interest. Specific sequences and the degree of binding is quantitated by directly counting the radioactive emissions.

Where the target nucleic acid is not amplified, detection using an appropriate hybridization probe may be performed directly on the separated nucleic acid. In those instances where the target nucleic acid is amplified, detection with the appropriate hybridization probe would be performed after amplification.

For the most part, the probe will be detectably labeled with an atom or inorganic radical, most commonly using radionuclides, but also heavy metals can be used. Conveniently, a radioactive label may be employed. Radioactive labels include ^{32}P, ^{125}I, ^{3}H, ^{14}C, ^{111}In, ^{99}Tc, or the like. Any radioactive label may be employed which provides for an adequate signal and has sufficient half-life. Other labels include ligands, which can serve as a specific binding pair member for a labeled ligand, and the like. A wide variety of labels routinely employed in immunoassays can readily be employed in the present assay. The choice of the label will be governed by the effect of the label on the rate of hybridization and binding of the probe to a nucleotide sequence. It will be necessary that the label provide sufficient sensitivity to detect the amount of a nucleotide sequence available for hybridization.

The manner in which the label is bound to the probe will vary depending upon the nature of the label. For a radioactive label, a wide variety of techniques can be employed. Commonly employed is nick translation with an a ^{32}P-dNTP or terminal phosphate hydrolysis with alkaline phosphatase followed by labeling with radioactive ^{32}P employing ^{32}P-NTP and T4 polynucleotide kinase. Alternatively, nucleotides can be

synthesized where one or more of the elements present are replaced with a radioactive isotope, *e.g.*, hydrogen with tritium. If desired, complementary labeled strands can be used as probes to enhance the concentration of hybridized label.

Standard hybridization techniques for detecting a nucleic acid sequence are known in the art. The particular hybridization technique is not essential to the invention. Other hybridization techniques are described by Gall and Pardue, Proc. Natl. Acad. Sci. 63:378, 1969); and John, *et al.*, Nature, 223:582, 1969). As improvements are made in hybridization techniques they can readily be applied in the method of the invention.

The amount of labeled probe present in the hybridization solution will vary widely, depending upon the nature of the label, the amount of the labeled probe that can reasonably bind to the filter, and the stringency of the hybridization. Generally, substantial excess over stoichiometric concentrations of the probe will be employed to enhance the rate of binding of the probe to the fixed target nucleic acid.

Currently a number of retroviral vectors used for human gene therapy are replication-defective and must be produced in "packaging cells," which contain integrated wild type virus genome sequences and thus provide all of the structural elements necessary to assemble viruses (*e.g.*, the *gag*, *pol*, and *env* gene products), but cannot encapsidate their own wild type virus genomes due to a deletion of the packaging signal sequence (*psi*). Replication-defective virus vectors created by removal of the viral structural genes and replacement with therapeutic genes are introduced into the packaging cells; so long as these vectors contain the *psi* signal, they can take advantage of the structural proteins provided by the cells and be encapsidated into virion. However, after infection of a target cell, the vectors are incapable of secondary horizontal infections of adjacent cells due to the deletion of the essential viral genes.

Accordingly, in one embodiment of the invention a replication incompetent JSRV vector is provided. The replication incompetent JSRV vector has one or more sequences associated with gag, pol, or env gene products mutated, deleted or otherwise rendered non-functional. In addition, the replication incompetent JSRV can include a heterologous polynucleotide sequence. The heterologous polynucleotide

sequence can encode a protein of interest, a therapeutic protein, a marker or any other sequence commonly used in protein delivery or therapeutic treatments. The heterologous nucleic acid sequence is operably linked to a regulatory nucleic acid sequence. As used herein, the term "heterologous" nucleic acid sequence refers to a sequence that originates from a foreign species, or, if from the same species, it may be substantially modified from its original form. Alternatively, an unchanged nucleic acid sequence that is not normally expressed in a cell is a heterologous nucleic acid sequence. The term "operably linked" refers to functional linkage between the regulatory sequence and the heterologous nucleic acid sequence. Typically, the heterologous sequence is linked to a promoter, resulting in a chimeric gene. The heterologous nucleic acid sequence is preferably under control of either the viral LTR promoter-enhancer signals or of an internal promoter, and retained signals within the retrovirual LTR can still bring about efficient integration of the vector into the host cell genome.

In another embodiment, a JSRV packaging system is provided. The packaging system includes a defective JSRV, as described above (*e.g.*, lacking a functional env, pol, gag, or combination thereof), and a host cell containing functional JSRV env, pol, gag or a combination thereof. The JSRV complement sequence present in the packaging cell line allow encapsidation of the defective JSRV into virions. Examples of packaging systems, e.g., lentiviral packaging systems are known in the art. Accordingly, one of skill in the art can modify the packaging systems for use with the JSRV of the invention. In addition, it is contemplated that transient co-transfection of packaging plamsids (e.g., plasmids containing gag, pol, env, or combination thereos) and vector plasmid into the same cell (*e.g.*, a 293T cell) can be used to propagate a defective JSRV of the invention.

The use of replication-defective vectors has been an important safeguard against the uncontrolled spread of virus, as replication-competent retroviruses have been shown to cause malignancies in primates (Donahue *et al.*, J. Exp. Med., 1992, 176:1124-1135). However, replication-defective retroviral vectors are produced from the packaging cells at titers on the order of only 10^{6-7} colony-forming units (cfu) per ml, which is barely adequate for transduction *in vivo*. In fact, clinical trials for gene

therapy of glioblastoma multiforme, a highly malignant brain tumor, have encountered major problems in achieving adequate levels of tumor cell transduction, and despite promising initial results in animal studies (Culver *et al.*, Science, 1992, 256:1550-1552). In order to increase transduction levels as much as possible, instead of using a single shot of virus-containing supernatant, the virus packaging cell line PA317 itself was injected into the brain tumors to constitutively produce retrovirus vectors carrying the HSV-tk gene (Oldfield *et al.*, Human Gene Therapy, 1993, 4:39-69). Subsequently, the protocol was further modified to include a debulking procedure followed by multiple injection sites, as it was found that the virus vectors did not diffuse far enough from the site of initial injection. Despite these modifications, the transduction efficiency has been estimated to less than 1% of the tumor cell mass and any significant tumor destruction is presumed to be due to the potent "bystander" effect of the HSV-tk/ganciclovir treatment. Thus efficient transduction of cancer cells in a solid tumor mass represents a major problem for cancer gene therapy.

As mentioned above, the integrated DNA intermediate is referred to as a provirus. Prior gene therapy or gene delivery systems use methods and retroviruses that require transcription of the provirus and assembly into infectious virus while in the presence of an appropriate helper virus or in a cell line containing appropriate sequences enabling encapsidation without coincident production of a contaminating helper virus. It is contemplated, however, that due to the specificity of the JSRV genome and its regulation within specific tissues a helper virus is not required for the production of the recombinant retrovirus of the invention for gene delivery or therapy.

The retroviral genome and the proviral DNA of the present invention have at least the following genes: the gag, the pol, and the env, which are flanked by two long terminal repeat (LTR) sequences containing cis-acting sequences such as *psi*. The *gag* gene encodes the internal structural (matrix, capsid, and nucleocapsid) proteins; the *pol* gene encodes the RNA-directed DNA polymerase (reverse transcriptase), protease and integrase; and the *env* gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of the virion RNAs. The LTR contains all other cis-acting sequences necessary for viral replication.

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the *psi* site). If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virion) are missing from the viral genome, the result is a cis defect which prevents encapsidation of genomic viral RNA. This type of modified vector is what has typically been used in prior gene delivery systems (*i.e.*, systems lacking elements which are required for encapsidation of the virion).

In a first embodiment, the invention provides a recombinant retrovirus capable of infecting a non-dividing cell, a dividing cell, or a cell having a cell proliferative disorder. The recombinant replication competent retrovirus of the present invention comprises a polynucleotide sequence having a viral GAG, a viral POL, a viral ENV, a heterologous polynucleotide and LTRs having sequences (or fragments thereof) as set forth in accession no. AF105220. For example, non-dividing cells of the lung can be infected using a modified JSRV comprising, for example, env and LTR's from JSRV, and additional sequence from lentiviral vectors known in the art.

The heterologous nucleic acid sequence is operably linked to a regulatory nucleic acid sequence. As used herein, the term "heterologous" nucleic acid sequence or "transgene" refers to a sequence that does not normally exist in the wild-type retrovirus or a sequence that originates from a foreign species, or, if from the same species, it may be substantially modified from its original form. Alternatively, an unchanged nucleic acid sequence that is not normally expressed in a cell is a heterologous nucleic acid sequence.

Depending upon the intended use of the retroviral vector of the present invention any number of heterologous polynucleotide or nucleic acid sequences may be inserted into the retroviral vector. For example, for *in vitro* studies commonly used marker genes or reporter genes may be used, including, antibiotic resistance and fluorescent molecules (*e.g.*, GFP). Additional polynucleotide sequences encoding any desired polypeptide sequence may also be inserted into the vector of the present

invention. Where *in vivo* delivery of a heterologous nucleic acid sequence is sought both therapeutic and non-therapeutic sequences may be used. For example, the heterologous sequence can encode a therapeutic molecule including antisense molecules or ribozymes directed to a particular gene associated with a cell proliferative disorder, the heterologous sequence can be a suicide gene (*e.g.*, HSV-tk or PNP), or a therapeutic protein (*e.g.*, Factor IX). Other therapeutic proteins applicable to the present invention are easily identified in the art (see for example, R. Crystal, Science 270:404-410 (1995)). Thus, the recombinant virus of the invention is capable of transferring a heterologous sequence or transgene into a target cell.

The term "regulatory nucleic acid sequence" refers collectively to promoter sequences, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell. One skilled in the art can readily identify regulatory nucleic acid sequence from public databases and materials. Furthermore, one skilled in the art can identify a regulatory sequence that is applicable for the intended use, for example, *in vivo*, *ex vivo*, or *in vitro*.

The heterologous sequence is typically linked to a promoter, resulting in a chimeric gene. The heterologous nucleic acid sequence is preferably under control of either the viral LTR promoter-enhancer signals or of an internal promoter, and retained signals within the retroviral LTR can still bring about efficient integration of the vector into the host cell genome. Accordingly, the recombinant retroviral vectors of the invention, the desired sequences, genes and/or gene fragments can be inserted at several sites and under different regulatory sequences. For example, a site for insertion can be the viral enhancer/promoter proximal site (*i.e.*, 5' LTR-driven gene locus). Alternatively, the desired sequences can be inserted into a regulatory sequence distal to the 5' LTR (*e.g.*, an IRES sequence 3' to the *gag* gene). Other distal sites include viral promoter sequences, where the expression of the desired sequence or

sequences is through splicing of the promoter proximal cistron, an internal heterologous promoter as SV40 or CMV, or an internal ribosome entry site (IRES).

An example of a heterologous polynucleotide sequence which may be used in accordance with the invention include green fluorescent protein (GFP) or a selectable marker gene. Marker genes are utilized to assay for the presence of the vector, and thus, to confirm infection and integration. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances, *e.g.*, histidinol, puromycin, hygromycin, neomycin, methotrexate, and other reporter genes known in the art. Other heterologous sequences include, for example, suicide genes, such as PNP and HSV-thymidine kinase, polynucleotide sequences that encode an antisense molecule, or polynucleotides sequences that encode a ribosome.

It can be advantageous to have at one's disposal more efficacious gene therapy vectors capable, in particular, of producing several proteins of interest efficiently. However, the presence of several promoters within the same vector very often manifests itself in a reduction or even a loss of expression over time. This is due to a well-known phenomenon of interference between promoter sequences. In this context, the publication of International Application WO93/03143 proposes a solution to this problem which consists in employing an IRES. It describes a dicistonic retroviral vector for the expression of two genes of interest placed under the control of the same promoter. For example, the presence of a picornavirus IRES site between these genes permits the production of the expression product originating from the second gene of interest by internal initiation of the translation of the dicistronic mRNA (see Morgan *et al.*, Nucleic Acids Research, 20:(6) 1293-1299 (1992)).

Normally, the entry of ribosomes into messenger RNA takes place via the cap located at the 5' end of all eukaryotic mRNAs. However, there are exceptions to this universal rule. The absence of a cap in some viral mRNAs suggests the existence of alternative structures permitting the entry of ribosomes at an internal site of these RNAs. To date, a number of these structures, designated IRES on account of their function, have been identified in the 5' noncoding region of uncapped viral mRNAs, such as that, in particular, of picornaviruses such as the poliomyelitis virus (Pelletier

et al., 1988, Mol. Cell. Biol., 8, 1103-1112) and the EMCV virus (encephalo-myocarditis virus (Jang *et al.*, J. Virol., 1988, 62, 2636-2643).

In another embodiment, a targeting polynucleotide sequence is included as part of the recombinant retroviral vector of the present invention. Preferably, the targeting ligand is operably linked to the env protein of the retrovirus, creating a chimeric retroviral env protein. The viral GAG, viral POL and viral ENV proteins can be derived from any suitable retrovirus (*e.g.*,MLV or lentivirus-derived) (Han *et al.*, Proc Natl Acad Sci USA, 1995, 92(21):9747-51). In another embodiment, the viral ENV protein is non-retrovirus-derived (*e.g.*, CMV or VSV).

The recombinant retrovirus of the invention is to effective for targeting expression in pulmonary tissue or a particular cell pulmonary cell type. In addition, other modification to the JSRV can be made to allow targeting to other cell types, including, for example, smooth muscle cells, hepatic cells, renal cells, fibroblasts, keratinocytes, mesenchymal stem cells, bone marrow cells, chondrocyte, epithelial cells, intestinal cells, neoplastic cells and others known in the art) such that the modified JSRV genome (*e.g.*, modified to contain a heterologous sequence) is delivered to a target non-dividing, a target dividing cell, or a target cell having a cell proliferative disorder.

Targeting can be achieved in two ways. The first way directs the retrovirus to a target cell by preferentially binding to cells having a molecule on the external surface of the cell. This method of targeting the recombinant JSRV utilizes expression of a targeting ligand on the coat of the recombinant JSRV to assist in targeting the virus to cells or tissues that have a receptor or binding molecule which interacts with the targeting ligand on the surface of the retrovirus. After infection of a cell by the virus, the virus injects its nucleic acid into the cell and the retrovirus genetic material can integrate into the host cell genome. The second method for targeting uses cell- or tissue- specific LTR to preferentially promote expression and transcription of the viral genome in a targeted cell which actively utilizes the regulatory elements, as described more fully below. The transferred retrovirus genetic material is then transcribed and translated into proteins within the host cell.

By inserting a heterologous nucleic acid sequence of interest into the viral vector of the invention, along with another gene which encodes, for example, the ligand for a receptor on a specific target cell, the vector is now target specific. Viral vectors can be made target specific by attaching, for example, a sugar, a glycolipid, or a protein (Han *et al.*, Proc Natl Acad Sci USA, 1995, 92(21):9747-51). Targeting can be accomplished by using an antibody to target the viral vector. Those of skill in the art will know of, or can readily ascertain, specific polynucleotide sequences which can be inserted into the viral genome or proteins which can be attached to a viral envelope to allow target specific delivery of the viral vector containing the nucleic acid sequence of interest.

Thus, the present invention, includes in one embodiment, a chimeric env protein comprising a retroviral env protein operably linked to a targeting polypeptide. The targeting polypeptide can be a cell specific receptor molecule, a ligand for a cell specific receptor, an antibody or antibody fragment to a cell specific antigenic epitope or any other ligand easily identified in the art which is capable of binding or interacting with a target cell. Examples of targeting polypeptides or molecules include bivalent antibodies using biotin-streptavidin as linkers (Etienne-Julan *et al.*, J. Of General Virol., 73:3251-3255, 1992); Roux *et al.*, Proc. Natl. Acad. Sci USA 86:9079-9083, 1989), recombinant virus containing in its envelope a sequence encoding a single-chain antibody variable region against a hapten (Russell *et al.*, Nucleic Acids Research, 21:1081-1085, 1993)), cloning of peptide hormone ligands into the retrovirus envelope (Kasahara *et al.*, Science, 266:1373-1376, 1994), chimeric EPO/env constructs (Kasahara *et al.*, 1994), single-chain antibody against the low density lipoprotein (LDL) receptor in the ecotropic MLV envelope, resulting in specific infection of HeLa cells expressing LDL receptor (Somia *et al.*, Proc. Natl. Acad. Sci USA, 92:7570-7574, 1995), and Dornberg and co-workers (Chu and Dornburg, J. Virol 69:2659-2663, 1995) have reported tissue-specific targeting of spleen necrosis virus (SNV), an avian retrovirus, using envelopes containing single-chain antibodies directed against tumor markers.

The invention provides a method of producing a recombinant JSRV capable of infecting a target cell comprising transfecting a suitable host cell with the following: a modified JSRV comprising a polynucleotide sequence encoding a viral gag, a viral pol and a viral env, a heterologous polynucleotide sequence, operably linked to a regulatory nucleic acid sequence, and recovering the recombinant virus.

Accordingly, the invention provides replication incompetent JSRV vectors and replication competent JSRV vectors that do not require helper virus or additional nucleic acid sequence or proteins in order to propagate and produce virion. For example, the nucleic acid sequences of the JSRV as set forth in accession no. AF105220 encodes, for example, a group specific antigen and reverse transcriptase, (and integrase and protease-enzymes necessary for maturation and reverse transcription), respectively, as discussed above. A JRSV vector contains a gag and pol which can be modified from known gag proteins of other retroviral vectors. In addition, the nucleic acid genome of the JSRV vector of the present invention includes a sequence encoding a viral envelope (ENV) protein. The env gene can be derived from any retroviruses. The env may be an amphotropic envelope protein which allows transduction of cells of human and other species or can be species specific. Further, it may be desirable to target the JSRV vector by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. As mentioned above, retroviral vectors can be made target specific by inserting, for example, a glycolipid, or a protein. Targeting is often accomplished by using an antibody to target the retroviral vector to an antigen on a particular cell-type (*e.g.*, a cell type found in a certain tissue, or a cancer cell type). Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific methods to achieve delivery of a retroviral vector to a specific target. Examples of retroviral-derived env genes include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), human immunodeficiency virus (HIV) and Rous Sarcoma Virus (RSV). Other env genes such as Vesicular stomatitis virus (VSV) (Protein G), cytomegalovirus envelope (CMV), or influenza virus hemagglutinin (HA) can also be used.

In another embodiment, the present invention provides JSRV vectors that are target specific due to LTR tissue specificity. Accordingly, in one embodiment, the present invention provides a JSRV having tissue-specific LTR elements at the 5' and 3' end of the retroviral genome.

The LTRs of JSRV allow for preferential expresson in pulmonary tissues and cell types. Accordingly, the JSRV LTR's are capable of driving transcription of a gene in one tissue while remaining largely "silent" in other tissue types. It will be understood, however, that tissue-specific LTRs may have a detectable amount of "background" or "base" activity in those tissues where they are silent. The degree to which a promoter is selectively activated in a target tissue can be expressed as a selectivity ratio (activity in a target tissue/activity in a control tissue). In this regard, a tissue specific LTR useful in the practice of the present invention typically has a selectivity ratio of greater than about 3. Preferably, the selectivity ratio is greater than about 15.

Tissue-specific LTRs or regulatory elements may be derived, for example, from genes or viruses that are differentially expressed in different tissues. For example, a variety of promoters have been identified which are suitable for up regulating expression in cardiac tissue. Included, for example, are the cardiac I-myosin heavy chain (AMHC) promoter and the cardiac I-actin promoter. Other examples of tissue-specific regulatory elements include, tissue-specific promoters, such as milk-specific (whey), pancreatic (insulin or elastase), actin promoter in smooth muscle cells or neuronal (myelin basic protein) promoters. Through the use of promoters, such as milk-specific promoters, recombinant retroviruses may be isolated directly from the biological fluid of the progeny.

In addition, numerous gene therapy methods, that take advantage of retroviral vectors, for treating a wide variety of diseases are well-known in the art (see, *e.g.*, U.S. Pat. Nos. 4,405,712 and 4,650,764; Friedmann, 1989, Science, 244:1275-1281; Mulligan, 1993, Science, 260:926-932, R. Crystal, 1995, Science 270:404-410, each of which are incorporated herein by reference in their entirety). An increasing number

of these methods are currently being applied in human clinical trials (Morgan, R., 1993, BioPharm, 6(1):32-35; see also, The Development of Human Gene Therapy, Theodore Friedmann, Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999. ISBN 0-87969-528-5, which is incorporated herein by reference in its entirety). The safety of these currently available gene therapy protocols can be substantially increased by using JSRV vectors of the present invention. For example, where the retroviral vector infects a non-targeted cell (*e.g.*, a non-pulmonary cell type), the retroviral genome will integrate but will not be transcribed. However, when the retroviral vector containing a tissue specific LTR or regulatory element infects a targeted cell the active tissue specific promoter will result in transcription and translation of the viral genome.

The phrase "non-dividing" cell refers to a cell that does not go through mitosis. Non-dividing cells may be blocked at any point in the cell cycle, (*e.g.*, G0/G1, G1/S, G2/M), as long as the cell is not actively dividing. For *ex vivo* infection, a dividing cell can be treated to block cell division by standard techniques used by those of skill in the art, including, irradiation, aphidocolin treatment, serum starvation, and contact inhibition. However, it should be understood that *ex vivo* infection is often performed without blocking the cells since many cells are already arrested (*e.g.*, stem cells). For example, a recombinant JSRV vector of the invention is capable of infecting a non-dividing cell, regardless of the mechanism used to block cell division or the point in the cell cycle at which the cell is blocked. Examples of pre-existing non-dividing cells in the body include neuronal, muscle, liver, certain epithelial cells (*e.g.*, skin), heart, lung, and bone marrow cells, and their derivatives.

By "dividing" cell is meant a cell that undergoes active mitosis, or meiosis. Such dividing cells include stem cells, skin cells (*e.g.*, fibroblasts and keratinocytes), gametes, and other dividing cells known in the art. Of particular interest and encompassed by the term dividing cell are cells having cell proliferative disorders, such as neoplastic cells. The term "cell proliferative disorder" refers to a condition characterized by an abnormal number of cells. The condition can include both hypertrophic (the continual multiplication of cells resulting in an overgrowth of a cell population within a tissue) and hypotrophic (a lack or deficiency of cells within a

tissue) cell growth or an excessive influx or migration of cells into an area of a body. The cell populations are not necessarily transformed, tumorigenic or malignant cells, but can include normal cells as well. Cell proliferative disorders include disorders associated with an overgrowth of connective tissues, such as various fibrotic conditions, including scleroderma, arthritis and liver cirrhosis. Cell proliferative disorders include neoplastic disorders such as head and neck carcinomas. Head and neck carcinomas would include, for example, carcinoma of the mouth, esophagus, throat, larynx, thyroid gland, tongue, lips, salivary glands, nose, paranasal sinuses, nasopharynx, superior nasal vault and sinus tumors, esthesioneuroblastoma, squamous call cancer, malignant melanoma, sinonasal undifferentiated carcinoma (SNUC) or blood neoplasia. Also included are carcinoma's of the regional lymph nodes including cervical lymph nodes, prelaryngeal lymph nodes, pulmonary juxtaesophageal lymph nodes and submandibular lymph nodes (Harrison's Principles of Internal Medicine (eds., Isselbacher, *et al.*, McGraw-Hill, Inc., 13th Edition, pp1850-1853, 1994). Other cancer types, include, but are not limited to, lung cancer, colon-rectum cancer, breast cancer, prostate cancer, urinary tract cancer, uterine cancer lymphoma, oral cancer, pancreatic cancer, leukemia, melanoma, stomach cancer and ovarian cancer.

The present invention also provides gene therapy for the treatment of cell proliferative disorders. Such therapy would achieve its therapeutic effect by introduction of an appropriate therapeutic polynucleotide sequence (*e.g.*, antisense, ribozymes, suicide genes), into cells of subject having the proliferative disorder. Delivery of polynucleotide constructs can be achieved using the recombinant retroviral vector of the present invention.

In addition, the therapeutic methods (*e.g.*, the gene therapy or gene delivery methods) as described herein can be performed *in vivo* or *ex vivo*. It may be preferable to remove the majority of a tumor prior to gene therapy, for example surgically or by radiation.

Thus, the invention provides a recombinant retrovirus capable of infecting a non-dividing cell, a dividing cell or a neoplastic cell comprising a JSRV GAG; POL;

ENV; a heterologous nucleic acid sequence operably linked to a regulatory nucleic acid sequence; and cis-acting nucleic acid sequences necessary for packaging, reverse transcription and integration.

The invention also provides a method of nucleic acid transfer to a target cell to provide expression of a particular nucleic acid sequence (*e.g.*, a heterologous sequence). Therefore, in another embodiment, the invention provides a method for introduction and expression of a heterologous nucleic acid sequence in a target cell comprising infecting the target cell with a recombinant JSRV vector containing a heterologous sequence and expressing the heterologous nucleic acid sequence in the target cell. As mentioned above, the target cell can be any cell type including dividing, non-dividing, neoplastic, immortalized, modified and other cell types recognized by those of skill in the art, so long as they are capable of infection by a retrovirus. Simply measuring expression of a detectable marker after contacting the cell with a recombinant JSRV of the invention is sufficient to ascertain infectivity. An example of a detectable marker is GFP. Examples of therapeutic heterologous sequence include sequence which modify the expression of chloride channels that are defective in cystic fibrosis by providing replacement sequence or sequence which modify or inhibit expression of the defective sequences (e.g., antisense sequences).

It may be desirable to modulate the expression of a gene in a cell by the introduction of a nucleic acid sequence (*e.g.*, the heterologous nucleic acid sequence) by the method of the invention, wherein the nucleic acid sequence give rise, for example, to an antisense or ribozyme molecule. The term "modulate" envisions the suppression of expression of a gene when it is over-expressed, or augmentation of expression when it is under-expressed. Where a cell proliferative disorder is associated with the expression of a gene, nucleic acid sequences that interfere with the gene's expression at the translational level can be used. This approach utilizes, for example, antisense nucleic acid, ribozymes, or triplex agents to block transcription or translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or triplex agent, or by cleaving it with a ribozyme.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, Scientific American, 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target cell. The use of antisense methods to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, Anal.Biochem., 172:289, 1988).

The antisense nucleic acid can be used to block expression of a mutant protein or a dominantly active gene product, such as amyloid precursor protein that accumulates in Alzheimer's disease. Such methods are also useful for the treatment of Huntington's disease, hereditary Parkinsonism, and other diseases. Of particular interest are the blocking of genes associated with cell-proliferative disorders. Antisense nucleic acids are also useful for the inhibition of expression of proteins associated with toxicity.

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher, *et al.*, Antisense Res. and Dev., 1(3):227, 1991; Helene, C., Anticancer Drug Design, 6(6):569, 1991).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, J.Amer.Med. Assn., 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

It may be desirable to transfer a nucleic acid encoding a biological response modifier. Included in this category are immunopotentiating agents including nucleic acids encoding a number of the cytokines classified as "interleukins". These include, for example, interleukins 1 through 12. Also included in this category, although not necessarily working according to the same mechanisms, are interferons, and in particular gamma interferon (γ-IFN), tumor necrosis factor (TNF) and granulocyte-macrophage-colony stimulating factor (GM-CSF). Other polypeptides include, for example, angiogenic factors and anti-angiogenic factors. It may be desirable to deliver such nucleic acids to bone marrow cells or macrophages to treat enzymatic deficiencies or immune defects. Nucleic acids encoding growth factors, toxic peptides, ligands, receptors, or other physiologically important proteins can also be introduced into specific target cells.

For diseases due to deficiency of a protein product, gene transfer could introduce a normal gene into the affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For example, it may be desirable to insert a factor which requires oxygen or ambient air for activiation such that it is expressed preferentially in the pulmonary tissue using the JSRV vector of the invention.

The present invention also provides gene therapy for the treatment of cell proliferative or immunologic disorders. Such therapy would achieve its therapeutic effect by introduction of an antisense or dominant negative encoding polynucleotide into cells having the proliferative disorder, wherein the polynucleotide binds to and prevents translation or expression of a gene associated with a cell-proliferative disorder. Delivery of heterologous nucleic acids useful in treating or modulating a cell proliferative disorder (*e.g.*, antisense polynucleotides) can be achieved using a JSRV vector of the present invention.

In another embodiment, the invention provides a method of treating a subject having a cell proliferative disorder. The subject can be any mammal, and is preferably a human. The subject is contacted with a recombinant replication competent or incompetent JSRV vector of the present invention. The contacting can

be *in vivo* or *ex vivo*. Methods of administering the JSRV vector of the invention are known in the art and include, for example, systemic administration, topical administration, intraperitoneal administration, intra-muscular administration, as well as administration directly at the site of a tumor or cell-proliferative disorder and other routes of administration known in the art.

Thus, the invention includes various pharmaceutical compositions useful for treating a cell proliferative disorder, preferably a pulmonary proliferative disorder. The pharmaceutical compositions according to the invention are prepared by bringing a JSRV vector containing a heterologous polynucleotide sequence useful in treating or modulating a cell proliferative disorder according to the present invention into a form suitable for administration to a subject using carriers, excipients and additives or auxiliaries. Frequently used carriers or auxiliaries include magnesium carbonate, titanium dioxide, lactose, mannitol and other sugars, talc, milk protein, gelatin, starch, vitamins, cellulose and its derivatives, animal and vegetable oils, polyethylene glycols and solvents, such as sterile water, alcohols, glycerol and polyhydric alcohols. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described, for instance, in Remington's Pharmaceutical Sciences, 15th ed. Easton: Mack Publishing Co., 1405 1412, 1461-1487 (1975) and The National Formulary XIV., 14th ed. Washington: American Pharmaceutical Association (1975), the contents of which are hereby incorporated by reference. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art. See Goodman and Gilman's The Pharmacological Basis for Therapeutics (7th ed.).

In some instances it may be advantageous to deliver and express a JSRV sequence of the invention locally (*e.g.*, within a particular tissue or cell type). For example, local expression of JSRV in certain lung tissues of an animal. The nucleic sequence may be directly delivered to the tissue and cells, for example. Such delivery methods are known in the art and include electroporation, viral vectors and direct DNA uptake.

For example, one type of nucleic acid delivery vehicle comprises liposomal transfection vesicles, including both anionic and cationic liposomal constructs. The use of anionic liposomes requires that the nucleic acids be entrapped within the liposome. Cationic liposomes do not require nucleic acid entrapment and instead may be formed by simple mixing of the nucleic acids and liposomes. The cationic liposomes avidly bind to the negatively charged nucleic acid molecules, including both DNA and RNA, to yield complexes which give reasonable transfection efficiency in many cell types. See, Farhood *et al.* (1992) Biochem. Biophys. Acta. 1111:239-246, the disclosure of which is incorporated herein by reference. A particularly preferred material for forming liposomal vesicles is lipofectin which is composed of an equimolar mixture of dioleylphosphatidyl ethanolamine (DOPE) and dioleyloxypropyl-triethylammonium (DOTMA), as described in Felgner and Ringold (1989) Nature 337:387-388, the disclosure of which is incorporated herein by reference.

It is also possible to combine delivery systems. For example, Kahn *et al.* (1992), *supra.*, teaches that a retroviral vector may be combined in a cationic DEAE-dextran vesicle to further enhance transformation efficiency. It is also possible to incorporate nuclear proteins into viral and/or liposomal delivery vesicles to even further improve transfection efficiencies. See, Kaneda *et al.* (1989) Science 243:375-378, the disclosure of which is incorporated herein by reference.

Another targeted delivery system for polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al.*, Trends Biochem. Sci., 6:77, 1981). In addition to mammalian cells,

liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al.*, Biotechniques, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidyl-glycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand. In general, the compounds bound to the surface of the targeted delivery system will be ligands and receptors which will allow the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest which will bind to another compound, such as a receptor.

The agents useful in the method of the invention can be administered, for *in vivo* application, parenterally by injection or by gradual perfusion over time. Administration may be intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally. For *in vitro* studies the agents may be added or dissolved in an appropriate biologically acceptable buffer and added to a cell or tissue.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents and inert gases and the like.

In one embodiment, the invention provides a pharmaceutical composition useful for inducing an immune response to a JSRV or related retrovirus in an animal comprising an immunologically effective amount of a JSRV or JSRV antigen (*e.g.*, env protein) in a pharmaceutically acceptable carrier. The term "immunogenically effective amount," as used in describing the invention, is meant to denote that amount of antigen which is necessary to induce in an animal the production of an immune response to a JSRV or JSRV antigen. Env protein(s) are particularly useful in

sensitizing the immune system of an animal such that, as one result, an immune response is produced which ameliorates the effect of an infection by a JSRV or related viral particle.

The JSRV or JSRV antigen (*e.g.* env protein) can be administered parenterally by injection, rapid infusion, nasopharyngeal absorption, dermal absorption, and orally. Pharmaceutically acceptable carrier preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers for occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable forms for suspending the liposomes include emulsions, suspensions, solutions, syrups, and elixirs containing inert diluents commonly used in the art, such as purified water. Besides the inert diluents, such compositions can also include adjuvants, wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

It is also possible for the antigenic preparations containing the JSRV or JSRV protein of the invention to include an adjuvant. Adjuvants are substances that can be used to nonspecifically augment a specific immune response. Normally, the adjuvant and the antigen are mixed prior to presentation to the immune system, or presented separately, but into the same site of the animal being immunized. Adjuvants can be loosely divided into several groups based on their composition. These groups include oil adjuvants (for example, Freund's Complete and Incomplete), mineral salts (for example, $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)$, silica, alum, $Al(OH)_3$, $Ca_3(PO_4)_2$, kaolin, and carbon), polynucleotides (for example, poly IC and poly AU acids), and certain natural substances (for example, wax D from Mycobacterium tuberculosis, as well as substances found in *Corynebacterium parvum*, *Bordetella pertussis*, and members of the genus *Brucella*).

In another embodiment, a method of inducing an immune response to an infectous JSRV in an animal is provided. Many different techniques exist for the

timing of the immunizations when a multiple immunization regimen is utilized. It is possible to use the antigenic preparation of the invention more than once to increase the levels and diversity of expression of the immune response of the immunized animal. Typically, if multiple immunizations are given, they will be spaced two to four weeks apart. Subjects in which an immune response to JSRV or a related retrovirus is desirable include sheep, swine, cattle and humans.

Generally, the dosage of JSRV or a JSRV protein administered to an animal will vary depending on such factors as age, condition, sex and extent of disease, if any, and other variables which can be adjusted by one of ordinary skill in the art.

The antigenic preparations of the invention can be administered as either single or multiple dosages and can vary from about 10 ug to about 1,000 ug for the antigen per dose, more preferably from about 50 ug to about 700 ug antigen per dose, most preferably from about 50 ug to about 300 ug antigen per dose.

It should be understood, that JSRV is typically administered in an inactive form in order to induce the immune response. Inactive forms of JSRV can include heat inactivated forms, as well as recombinant forms that lack the ability to replicate without a helper cell system, as described herein.

The following Examples are intended to illustrate, but not to limit the invention. While such Examples are typical of those that might be used, other procedures known to those skilled in the art may alternatively be utilized.

EXAMPLES

EXAMPLE 1

ISOLATION AND CLONING OF JSRV

Molecular cloning. Molecular cloning of JSRV proviral DNA was carried out using standard molecular biology and cloning techniques. The strategy is shown in Fig. 1. High-molecular-weight genomic DNA was isolated from a lung tumor collected from an adult sheep with naturally acquired SPA. The genomic DNA was digested to completion with *XbaI* (an enzyme believed not to cut within JSRV DNA

on the basis of the York *et al.* sequence), ligated to *XbaI*-digested Dash II phage vector (Stratagene), and packaged into phage particles by using Gigapack III gold-packaging extracts (Stratagene). The resulting phage library (750,000 PFU) was divided into 15 sublibraries, and each sublibrary was independently amplified. DNA was extracted from an aliquot of each sublibrary and subjected to PCR for JSRV provirus by using a JSRV U3-specific hemi-nested PCR (U3 hn-PCR) to discriminate between exogenous JSRV and endogenous JSRV-related proviruses. Of the 15 sublibraries, 3 were positive for exogenous JSRV sequences. The positive sublibraries also were tested for the presence of exogenous JSRV by PCR amplification of a portion of the *gag* region followed by digestion of the PCR product with *ScaI*. The *gag ScaI* site is a molecular marker for exogenous JSRV. Sublibrary 2 was then plated onto bacterial agar plates and subjected to hybridization of plaque lifts with two ^{32}P-labelled probes on replica filters: a JSRV long terminal repeat (LTR)-specific probe and a *gag*-specific probe. Under the hybridization conditions used, these probes hybridized with both endogenous and exogenous JSRV sequences. Primary plaques positive for both LTR and *gag* probes were picked, and DNA was extracted from a portion and screened for the presence of exogenous JSRV sequences by U3 hn-PCR. Exogenous JSRV-positive primary plaque picks were further purified by dilution and plating for isolated plaques on bacterial lawns, followed by hybridization with both LTR and *env* probes. A recombinant phage carrying a seemingly full-length JSRV provirus was subcloned into pBluescript (Stratagene) to give pJSRV$_{21}$. Both strands of p JSRV$_{21}$ were completely sequenced with an automated liquid fluorescence sequencer.

Isolation of JSRV$_{21}$. As described above, an integrated copy of JSRV provirus from DNA isolated from an animal with a spontaneous case of SPA was obtained. A lambda phage library from a lung tumor of an animal with SPA was screened by combining classical plaque hybridizations involving JSRV DNA probes with sib selection procedures. The sib selection involved PCR amplifications that could distinguish exogenous JSRV from multicopy endogenous JSRV-related sequences present in the sheep genome. This combination of techniques was useful because the available JSRV hybridization probes cross-hybridized with the endogenous JSRV-related sequences. The cloning strategy resulted in the isolation of a full-length

exogenous JSRV proviral clone ($JSRV_{21}$). The insert from this clone was subcloned into a plasmid to give p $JSRV_{21}$. The nucleotide sequence of $JSRV_{21}$ has been deposited in GenBank under accession no. AF105220.

Plasmid $pCMV2JS_{21}$ was generated by replacing the U3 region in the upstream LTR of pJSRV21 with the human cytomegalovirus (CMV) immediate-early promoter by methods known in the art (Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The CMV promoter was obtained by PCR amplification from the pCDNA3 plasmid (Invitrogen) with primers CMVNotIf (AAAGGGTTGCGGCCGCCGATGTAC GGGCCAGATATAC (SEQ ID NO:____)) and CMV-r2 (CAGAGAGCTCTGC TTATATAGACCTCCCAC (SEQ ID NO:____)) and the Pfu Turbo polymerase (Stratagene) under PCR conditions as suggested by the manufacturer. The resulting PCR product was cut with *NotI* and then ligated to an amplified portion of $JSRV_{21}$ spanning position 13 in the U3 to +618 in gag. This portion of $JSRV_{21}$, which includes R, U5, and the beginning of gag, was amplified by PCR with primers JS21-R (GCATTGTAATAAAGCAGAGTATCAGCC (SEQ ID NO:____)) and JS21663-r (GGAACCAAGGGCAAACTCCTCAATAAATGAA (SEQ ID NO:____)) and the Pfu Turbo polymerase as above. The ligation reaction mixture was reamplified by PCR with primers CMVNotIf and JS21663-r, and the resulting product was digested with NotI and PacI and inserted into NotI-PacI-digested $JSRV_{21}$ to give $pCMV2JS_{21}$ (FIG. 2).

Sequence analysis showed that $JSRV_{21}$ possesses the hallmarks of integrated retroviral proviruses, such as the presence of a CA-TG dinucleotide pair present at the termini of the upstream and downstream viral LTRs, the loss of 2 nucleotides (nt) from the termini of the LTRs during integration, and an apparent duplication of 6 nt of cellular flanking sequences (TGTGTC (SEQ ID NO:____)) at the integration site. The flanking cellular sequences in the JSRV21 clone were 393 and 1,006 bp long and did not align with known cellular sequences (including proto-oncogenes).

$JSRV_{21}$ provirus is 7,834 bp long, and the viral genome (R to R) is 7,455 nt. $JSRV_{21}$ shows the characteristic genomic organization of type D and type B retroviruses, with pro in a different open reading frame from pol (Fig. 2a). These results were consistent with the genomic organization of JSRV deduced by York *et al.* for JSRV-SA. $JSRV_{21}$ showed overall 93% homology to JSRV-SA. The homology was 90% in the LTRs (89% in U3), 91% in *gag*, 96% in *pol*, and 91% in *env*. $JSRV_{21}$ is 7 bp shorter than JSRV-SA and in particular has a 5-bp deletion in U3 with respect to JSRV-SA. One difference between the suspected coding region of JSRV-SA and $JSRV_{21}$ was in the *pro* region: the *pro* open reading frame in $JSRV_{21}$ starts 53 nt downstream from the putative *pro* start in JSRV-SA; in particular, there are two stop codons in $JSRV_{21}$ at positions 1919 and 1931 that are not present in JSRV-SA. Thus, for $JSRV_{21}$, the 1 translational frameshift that presumably occurs during synthesis of the gag-pro-pol polyprotein precursor must occur downstream of the stop codon at nt 1932. The gag protein sequences for these two viruses have 100% identity in the region shown, so that the differences in the pro sequences did not affect the overlapping gag gene product. The orf-x open reading frame first identified in JSRV-SA was conserved in pJSRV21, suggesting that it plays a functional role.

The presence of the *Sca*I site at position 1726 in *gag* and the nucleotide sequence of the U3 indicated that $JSRV_{21}$ has the molecular markers of an exogenous JSRV and that it was not an endogenous JSRV-related provirus.

In vitro transfections. 293T cells were grown in Dulbecco minimum essential medium supplemented with 10% fetal bovine serum in 10-cm tissue culture dishes at 37°C under 5% CO_2. The cells were transfected with 45 μg of $pCMV2JS_{21}$ DNA by using the CalPhos mammalian transfection kit (Clonetech) as recommended by the manufacturer. Medium was replaced after 12 to 16 h with 5 ml of fresh medium. The medium was then harvested at 24, 48, and 72 h after the first medium change. The medium was filtered through a 0.45-μm-pore-size filter, and virus was pelleted by ultracentrifugation through a double layer of glycerol (25 and 50%, vol/vol) at 100,000 × g for 1 h at 4°C. The viral pellet was resuspended in TNE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA) at a 300-fold-higher concentration with respect to the initial supernatant and stored at -140°C until further use.

Western blotting. Western blotting was performed on 15-μl aliquots of concentrated $JSRV_{21}$ particles obtained from 293T cells transiently transfected with $pCMV2JS_{21}$. A rabbit antiserum to JSRV major capsid protein (CA) was used essentially as described previously (Palmarini *et al.* 1995. J. Gen. Virol. 76:2731-2737; Sharp and Herring. 1983. J. Gen. Virol. 64:2323-2327), except that an enhanced chemiluminescence detection system (Supersignal; Pierce) was used as recommended by the manufacturer. Concentrated lung fluid collected from an animal with a natural case of SPA was prepared as described previously (Palmarini *et al.* 1995. J. Gen. Virol. 76:2731-2737; Sharp and Herring. 1983. J. Gen. Virol. 64:2323-2327) and used as a positive control.

In vivo DNA transfections. All the lambs used in this study were obtained from a maedi-visna virus-free flock raised at the Moredun Research Institute. Three newborn lambs were inoculated intratracheally with $pJSRV_{21}$ DNA complexed with a cationic lipid (GL-67) formulated with the neutral colipid DOPE in a molar ratio of 1:2. GL-67-DOPE was prepared as described in Lee *et al.* 1996. Hum. Gene Ther. 7:1701-1717. For each transfected animal, a total of 1 mg of $pJSRV_{21}$ DNA was complexed with GL-67-DOPE at the suggested molar ratio and diluted to a final volume of 5 ml with distilled water. Five animals kept in the high-security unit were used as uninoculated controls.

Peripheral blood mononuclear cells (PBMCs) were collected from transfected or control lambs at various times postinoculation (Table 1) and stored at -70°C. Two inoculated lambs and two uninoculated controls were killed 38 weeks postinoculation, and samples of lungs, mediastinal lymph nodes, spleens, and kidneys were collected. The tissues were split into two portions: the first portion was snap-frozen in liquid nitrogen and stored at -70°C for subsequent isolation of nucleic acids; the remainder was fixed in 10% neutral buffered formalin, processed routinely in an automatic tissue processor, embedded in paraffin wax, and sectioned into 4- to 6-μm-thick slices. Genomic DNA was prepared as described above.

PCR analysis. The presence of JSRV proviral DNA in PBMCs and tissues collected from the *in vivo*-transfected animals and from uninoculated control animals

was investigated by the use of a JSRV U3 hn-PCR as described in Palmarini *et al.* 1996. J. Gen. Virol. 77:2991-2998, except that for each sample, five 500-ng replicates of DNA were used (2.5 μg in total) and the samples were considered positive when one or more PCR replicate was positive. In each PCR assay, 5 to 10 500-ng replicates of calf thymus DNA were subjected to JSRV-specific U3 hn-PCR as additional negative controls.

In vivo infections. Four newborn lambs were inoculated intratracheally with 1 ml each of concentrated supernatant collected from 293T cells transiently transfected with $pCMV2JS_{21}$. The inoculum was diluted in phosphate-buffered saline (5 ml (final volume)) immediately before use. Two lambs were inoculated with phosphate-buffered saline alone and were kept as uninoculated controls. All the lambs were killed 4 months postinoculation, and tissues were treated as for the *in vivo*-transfected animals.

Assays for exogenous JSRV provirus in lungs and lung tumors of inoculated and control animals included exogenous virus-specific PCR in the U3 region of the LTR and testing for an exogenous virus-specific ScaI site in an LTR-gag hn-PCR product.

Histologic examination and immunohistochemistry. Lung sections (4 to 6 μm thick) were stained with hematoxylin and eosin and examined by light microscopy for tumor lesions. Sections were also examined for the presence of JSRV major capsid protein by immunohistochemistry as described in Palmarini*et al.* 1995. J. Gen. Virol. 76:2731-2737, except that an antigen retrieval step was included by microwaving the sections at 800 W twice for 7 min. SPA tumor tissue was used as a positive control.

In vivo DNA transfection of $pJSRV_{21}$. To test the infectivity of the $JSRV_{21}$ provirus, *in vivo* DNA transfection was performed in sheep. Three newborn black-face lambs were inoculated intratracheally with $pJSRV_{21}$ DNA complexed with a cationic lipid (GL-67-DOPE) that favors transfection of lung cells. PBMCs were collected at various times postinoculation (2 to 22 weeks), and the presence of JSRV provirus and transcripts was assessed by U3 hn-PCR (Table 1). All three lambs showed detectable JSRV sequences at various times postinoculation. The levels of

JSRV DNA in PBMCs from inoculated lambs were low, as judged by the fraction of replicate PCR products that scored positive. However, they were similar to the levels of JSRV DNA detected in PBMCs from lambs experimentally inoculated with concentrated SPA lung fluid. These results indicated that $pJSRV_{21}$ contained an infectious JSRV provirus. On the other hand, when two inoculated animals were sacrificed at 9 months postinfection, no SPA lesions were observed in the lungs by macroscopic or histologic examinations. JSRV antigens were not detected by immunohistochemistry for JSRV CA antigen in the lungs of the inoculated animals; only the highly sensitive U3 hn-PCR allowed the detection of JSRV provirus in the lungs of one inoculated animal and in the mediastinal lymph nodes and PBMCs of another (Table 1). As controls, PBMCs from five uninoculated control animals were tested by U3 hn-PCR, and they were always negative (none of five replicates were positive for each animal). In addition, two uninoculated animals were sacrificed and lung, mediastinal lymph node, spleen, and kidney samples were tested by U3 hn-PCR; they were also negative. In each PCR assay, 5 to 10 500-ng replicates of calf thymus DNA were subjected to JSRV-specific U3 hn-PCR as additional negative controls, and they were always negative.

TABLE 1. JSRV U3 hn-PCR on samples from *in vivo*-transfected lambs

	Presence of JSRV provirus[a]:							
Lamb	Antemortem[b]			Postmortem[c] in:				
	6wk	12wk	22wk	PBMC	Lungs	MLN[d]	Spleen	Kidney
71	4/5	1/5	1/5	ND[e]	ND	ND	ND	ND
73	4/5	1/5	1/5	0/5	5/5	0/5	0/5	0/5
74	5/5	4/5	1/5	0/5	0/5	1/5	0/5	0/5

[a] Number of positive reactions/total number of replicate reactions on 500 ng of DNA.

[b] Weeks postinfection. PBMC were used for these experiments.

[c] Animals were sacrificed for postmortem analysis at 38 weeks postinfection.

[d] MLN, mediastinal lymph nodes.

[e] ND, not done.

Synthesis of JSRV particles *in vitro*. Because no *in vitro* culture systems were available for JSRV, attempts were made to recover virus particles by direct transfection of a derivative of $pJSRV_{21}$ containing a simian virus 40 origin of replication into highly transfectable human 293T cells. This did not result in the production of detectable virus in the culture supernatants. It seemed possible that the JSRV LTR was not active in 293T cells, and so the U3 region of the upstream LTR in $pJSRV_{21}$ was replaced with the human CMV immediate-early promoter, which is highly active in these cells (Fig. 2b). The CMV promoter was positioned so that the resulting RNA transcript would be very similar to wild-type JSRV RNA. When the resulting plasmid ($pCMV2JS_{21}$) was transfected into 293T cells, substantial amounts of $JSRV_{21}$ virus were released into the supernatant. Western blot analysis for JSRV CA protein indicated that the amount of virions produced from transfected 293T cells was comparable to that present in lung fluid from SPA-affected sheep. Moreover, the fact that the supernatants from transfected 293T cells showed CA protein of the mature (cleaved) size strongly suggested that normal virion morphogenesis and polyprotein cleavage (presumably mediated by functional JSRV protease) took place. Enzymatically active RT could also be detected in the 293T cell supernatants by standard exogenous assays.

Analysis of $JSRV_{21}$ buoyant density. Approximately 700 µl of concentrated $JSRV_{21}$ particles was analyzed by isopycnic centrifugation on a linear 25 to 60% (wt/wt) continuous sucrose gradient in an SW41 rotor (Beckman) at 25,000 rpm for 16 h at 4°C. Fractions of approximately 450 µl were collected, and their density was determined by refractometry. Consecutive fractions were pooled two at a time and diluted with 10 ml of TNE buffer, and virus was recovered by centrifugation in an SW41 rotor at 35,000 rpm for 1 h at 4°C. Viral pellets were resuspended in 20 µl of TNE buffer and used in a conventional exogenous RT assay with poly(rA)-oligo(dT). For analysis of viral cores, 700 µl of concentrated JSRV was treated with a final concentration of 0.1% (vol/vol) Triton X-100 for 8 min at room temperature prior to density gradient analysis.

Supernatants from pCMV2JS$_{21}$-transfected 293T cells were analyzed by isopycnic centrifugation in sucrose density gradients. Supernatants from transfected cells contained RT activity that could be measured by an exogenous RT assay with poly(rA)-oligo(dT) as the template primer. RT assays across the sucrose gradient indicated a peak of RT activity with a buoyant density of approximately 1.15 g/ml (Fig. 3a). This was consistent with the buoyant density of retroviruses in general, although it was slightly lower than that reported for JSRV (1.16 to 1.18 g/ml) when the virus was isolated directly from the lung secretions of SPA-affected animals. Treatment of the 293T supernatants with 0.1% Triton X-100 prior to centrifugation shifted the RT peak to 1.218 to 1.238 g/ml, consistent with the release of viral cores (Fig. 3b) and suggesting that complete viral particles had been synthesized. Supernatants from mock-transfected 293T cells showed no RT activity.

Experimental induction of SPA. Four newborn black-face lambs were inoculated intratracheally with concentrated JSRV$_{21}$ stocks obtained from transfected 293T cells. At 4 months postinoculation, one of the animals showed clinical signs of respiratory distress suggestive of SPA. All four animals were sacrificed at this time.

At necropsy, the lungs of the clinically affected lamb showed gross pathological changes typical of SPA. They were both considerably enlarged and heavier than normal due to extensive lesions in the dependent areas of the cranial, medial, and caudal lobes. The lesions had a reddish translucent appearance and were well demarcated from the unaffected dorsal areas of the lungs, although some isolated small foci were scattered throughout the lungs. At the margins of the lesions, small reddish white nodules, approximately 3 to 5 mm in diameter, were observed. Transverse sections of the affected areas were clearly consolidated, and a moderate amount of clear, foamy fluid exuded from the cut surface and airways, as seen in naturally occurring and experimentally transmitted SPA. A few small foci with similar features also were observed in the caudal lobes of a second lamb. Histologic examination revealed the presence of multifocal neoplastic foci in both of these animals. Lesions comprised many small intra-alveolar and bronchiolar papilliform proliferations of cuboidal or prismatic epithelial cells. Some of these neoplastic nodules had an interstitial myxoid or fibrotic appearance. Alveoli adjacent to tumor

nodules contained a small number of alveolar macrophages. The above lesions were consistent with previously described features of SPA. To test if the tumors expressed JSRV protein, immunohistochemistry with an antiserum raised against JSRV CA protein was carried out. The tumor cells showed readily detectable staining for JSRV CA protein (reddish brown stain), while the surrounding normal tissue was negative for viral protein. As expected, two uninoculated control lambs showed no signs of disease, and at necropsy their lungs showed no signs of macroscopic or histologic SPA lesions, as well as no immunoreactive material.

To further test if the tumors in the $JSRV_{21}$-inoculated lambs resulted from exogenous viral infection, tumor tissue DNA was tested from the infected animals for the presence of exogenous $JSRV_{21}$ DNA sequences. PCR was carried out with primers from the U3 region of the LTR that are specific for exogenous JSRV. Lung tissues from both of the infected lambs with SPA lesions and from one of the infected animals that did not show SPA were positive for exogenous JSRV LTR sequences. As expected, no exogenous JSRV sequences were amplified from lung DNA from the uninoculated controls. To further confirm the presence of exogenous JSRV sequences in tumor DNA, an exogenous JSRV-specific *ScaI* site present in the gag region was measured by performing hn-PCR that favors amplification of exogenous JSRV sequences on tissue DNAs followed by digestion of the PCR product with *ScaI*. DNA from the tumor tissues of both lambs that developed SPA showed evidence of exogenous JSRV, as indicated by the appearance of more rapidly migrating *ScaI* cleavage products in the gel. These results indicated that both tumors induced by $JSRV_{21}$ infection contained exogenous JSRV sequences from the LTR and *gag* regions. This further supported the conclusion that $JSRV_{21}$ induced the SPA lesions in the infected animals.

It should be emphasized that the infectious $JSRV_{21}$ was obtained by transient transfection of $pCMV2JS_{21}$ plasmid DNA into human 293T cells. While the virus obtained by this technique is infectious and should be identical to that obtained from infected ovine tumor tissues (since the RNA transcribed in the transfected 293T cells is identical to genomic $JSRV_{21}$ RNA), there was an additional advantage. It has been shown that uninfected sheep cells carry several copies of highly related endogenous

JSRV DNA but that human DNA does not contain cross-hybridizing sequences. Thus, the potential for recombination between the exogenous $JSRV_{21}$ genome and ovine endogenous JSRV-related viruses during generation of $JSRV_{21}$ virus stocks was eliminated. Moreover, the fact that the virus was obtained by a transient transfection further minimized the likelihood of any low-level genetic interaction between the $JSRV_{21}$ genome and potential distantly related (nonhybridizing) endogenous viruses of humans.

Another area of interest is the strict association of JSRV expression with cells of the lungs. *In vivo*, JSRV infects several cell types, but viral antigens can be detected in great abundance only in the epithelial tumor cells of the lungs. To investigate whether the JSRV LTR contains enhancers specific for the cells in which the tumor originates (type II pneumocytes, Clara cells, and/or a common precursor) the experiments below were performed.

EXAMPLE 2

THE LONG TERMINAL REPEATS OF JAAGSIEKTE SHEEP RETROVIRUS (JSRV) ARE PREFERENTIALLY ACTIVATED IN TYPE II PNEUMOCYTES

Cell cultures. The cell lines used in this study, tissue, cells and animal species from which they originate and the source, reference or ATCC catalog number are listed in Table 2. Cells were grown at 37°C with 5% CO_2. MLE-15 cell line (provided by J. Whitsett), MLE-12, JS-7 and primary fetal lamb lung (FLL) cells were grown in RPMI 1640 (Gibco BRL), 2% fetal bovine serum (FBS), 0.5 % insulin-transferrin-sodium selenite (ITS) (Sigma) modified with the addition of 5mg/l transferrin, 10 mM Hepes, $1X10^{-8}$ M β-estradiol and $1X10^{-8}$ M Hydrocortisone. 293T cell line, OAT, CP-MRI, OA1, mtCC1-2 (provided by Dr. F. DeMayo), IC-21 and 3T3 were grown in DMEM (ATCC) and 10% FBS. IC-21 and ABI-2 were grown in RPMI 1640 (Gibco BRL) and 10% FBS. FOP, ST3, CP-ATCC, C2C12 and TCMK cell line were grown in DMEM (ATCC), 1X non-essential aminoacids (Cellgro) and 10% FBS. F9 cell line was grown in DMEM (ATCC), $7X10^{-6}$ M mercaptoethanol, 1X non-essential aminoacids and 10% FBS. BV2 and A549 cell lines were grown in F-12K (Gibco, BRL) and 10% FBS. H441 and H358 cell lines were grown in RPMI

1640 (Gibco BRL) adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 10 mM Hepes with 10%FBS.

Table 2. Cell lines

Cell Lines	Species of origin	Tissue/cell type	ATCC #/ Reference
A549	Human	Lung carcinoma	CCL-185
H441	Human	Bronchioloalveolar carcinoma	HTB-174
H358	Human	Bronchioloalveolar carcinoma	CRL-5807
293T	Human	Embryonic Kidney	Lebrowsky *et al.*
OA1	Sheep	Brain fibroblasts	CRL-6538
OAT	Sheep	Sheep testis	CRL-6546
FLL	Sheep	Primary fetal lamb	MRI*
JS7	Sheep	Bronchioloalveolar carcinoma	Jassim
CP-MRI	Sheep	Choroid plexus	MRI*
CP-ATCC	Sheep	Choroid plexus	CRL-1700
MtCC1-2	Mouse	Clara cells	Magdaleno *et al.*
BV2	Mouse	Microglia	Tenner A*
F9	Mouse	Testicular carcinoma	CRL-1720
FOP	Mouse	Mammary carcinoma	
IC-21	Mouse	Peritoneal macrophages	TIB-186
MHS	Mouse	Alveolar macrophages	CRL-2019
C2C12	Mouse	Myoblasts	CRL-1772
ABI-2	Mouse	Hybridoma	HB-33
MLE-12	Mouse	Lung epithelial	CRL-2110
TCMK	Mouse	Mouse Kidney	CCL-139
3T3	Mouse	Mouse embryo	CCL-92
MLE-15	Mouse	Type II pneumocytes	Wikenheiser *et al.*
ST3	Mouse	Thymus stroma	Brightman *et al.*

Oligonucleotides. For the electrophoretic mobility shift assays (EMSA) the following double stranded oligonucleotide probe were used: JS_{21}wt(-267/-247) (TGCGGGGGACGAC CCGTGAA (SEQ ID NO:___)) and JS_{21}mt(-267/-247) (TGCGGTTTACGACCCGTGAA (SEQ ID NO:____); mutated nucleotides are shown in bold). JS_{21}wt(-266/-247) corresponds to position –266 to –247 of the U3 of $JSRV_{21}$ and includes an NF-κB-like binding site (underlined). JS_{21}mt(-266/-247) has three nucleotides changes (in bold) with respect to JS_{21}wt(-267/-247) to alter the NF-κB-like site. Oligonucleotide probes for the consensus sequence of NF-κB were purchased from Geneka as positive controls.

Plasmids. Plasmids pGL3-control, pGL3-promoter and pGL3-basic where purchased from Promega. pGL3-control expresses the firefly luciferase gene (*luc*) under the control of the SV40 promoter and enhancer regions; pGL3-promoter expresses the *luc* gene under an SV40 promoter while pGL3-basic is devoided of eukaryotic promoter and enhancer regions. Plasmid pMLV-luc was obtained by inserting the whole LTR of Moloney-murine leukemia virus (M-MuLV) into pGL3-basic by PCR-based cloning techniques. PCRs were performed using the *Pfu-Turbo* polymerase (Stratagene) as recommended by the manufacturers. The LTR of M-MLV was amplified from plasmid p63.2. Plasmid pCMV-luc was derived by inserting the *HindIII-BamHI* fragment of pGL3-basic containing the *luc* gene and the poly(A) signal into pCDNA3.1 (Invitrogene).

The LTR of $JSRV_{21}$ were amplified from $pJSRV_{21}$ and inserted into the *MluI* and *BglII* site of pGL3-basic. The resulting plasmid was called pJS21-luc. The derivatives of pJS21-luc described below were all cloned into the *MluI* and *BglII* sites of pGL3-basic.

Progressive 5' deletions of pJS21-luc were generated by PCR cloning. All the various constructs (including those described below) were checked by nucleotide sequencing and/or restriction digestion.

The linker-scanning mutant pJS21(Δ-209/-167)-luc contains the whole JSRV LTR with the exception of a portion of the U3 encompassing nucleotides –209 and –166. Plasmid pJS21U3R-luc is composed of the U3 and R region of the pJS21 LTR.

Plasmid pJS21U5(+63)-luc is truncated in position +63 in the U5. Plasmid pNFKBm-luc was obtained by amplification of pJS21-luc with primers 3LTR-*BglII* and NFKBm*MluI*. Primer NFKBm*MluI* has incorporated in its sequence the desired mutation of the NF-κB-like binding site present at position –262 of pJS21-luc (GGG → TTT).

Plasmid pMLVp-luc was obtained by inserting the proximal promoter region of M-MLV (from –150 in the U3 until the end of U5) into pGL3-basic. Plasmid pMLVp+JSe was derived by inserting the $JSRV_{21}$ enhancers in the U3 region between –40 and –267 in front of pMLVp-luc. Plasmid pSVp+JSe was derived by inserting the U3 region of $JSRV_{21}$ between –51 and –267 into pGL3-promoter.

Plasmid pJSp+SVe has the SV40 enhancer driving the expression of the JSRV proximal promoter region starting at –51 in the U3 and includes the R and U5 region of the JSRV LTR. pJSp+SVe was derived by inserting the SV40 enhancer region from pGL3-control into pJS21(-51)-*luc*.

For the transactivation experiments the following expression plasmids were used. Plasmid pBETNFI-B1f expressing the NFI-A1.1 isoform driven by the chicken β-actin promoter and the control plasmid pBET containing only the β-actin promoter were provided by C. Bachurski and were originally developed by T. Tamura. Plasmids pCMV-TTFI, expressing the thyroid transcription factor (TTF-I) was originally made by R. Di Lauro and provided by G. Suske (Philipps-Universität, Margburg, Germany); pCMV-HNF3α and pCMV-HNF3β expressing the hepatocyte nuclear factor (HNF) 3α and β were originally developed by R.H. Costa and provided by G. Suske as was pEVR2-Sp1, expressing the Sp-1 transcription factor under the control of the CMV promoter. To adjust the luciferase values for transfection efficiency and lysate preparations the following plasmids were used: pCMV-βgal expressing the β-galactosidase gene under the control of the CMV promoter; pRL-tk (Promega) expressing the *renilla* luciferase under the control of the herpes simplex tymidine kinase promoter and pRL-null (Promega) a promoterless plasmid containing the *renilla* luciferase gene.

Transient transfections and luciferase assays. Transient transfections were performed on 2-4 X 10^5 cells plated on six well plates (Falcon) approximately 24 hours prior to transfection. For each well, 2 μg in total of plasmid DNA (1 μg of reporter plasmid and 1 μg of pCMV-βgal to adjust for transfection efficiency) and 6 μl of Fugene (Boehringer) were used as recommended by the manufacturers. In selected cell lines (MLE-15, mtCC1-2, 3T3, TCMK, ST3, CP-MRI and CP-ATCC) experiments were performed using the dual luciferase reporter system (Promega) (0.5 μg of reporter plasmid and 0.5 μg or 50ng of pRL-tk or pRL-null) and the activity of pJS21-luc was compared to that one of different neutral promoters (pGL3-control, pMLV-luc, pCMV-luc, pGL3-basic). For the transactivation experiments 200ng of pJS21-luc, 1 to 200ng of transactivating plasmid (or control plasmid containing the same promoter as the transactivating plasmid) and 100 ng of pRL-null was used. After 48 h, transfected cells were washed with PBS, lysed with 400 μl/well of 1 X Reporter Lysis Buffer (Promega), and frozen at -20°C. Luciferase assays were performed on 20 μl of the cleared lysate by rapid addition of Luciferase Assay Reagent (Promega) and light output was integrated over 10 sec at room temperature using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Luciferase activity was normalized for transfection efficiency and cell extract preparation by either assaying 50 μl of each lysate for β-galactosidase activity using the Luminescent β-galactosidase Genetic Reporter System II (Clontech) as recommended by the manufacturers, or measuring the *renilla* luciferase activity driven by pRL-tk and pRL-null in the Dual Luciferase System (Promega) as recommended by the manufacturers. The relative activity of pGL3-control adjusted for transfection efficiency was set to 100 for the experiments aimed to compare the activity of pJS21-luc across different cell lines.

In selected cell lines the activity of pJS21-luc was compared to different "neutral" promoters (pGL3-control, pCMV-luc, pMLV-luc or pGL3-basic) using either pCMV-βgal or pRL-tk or pRL-null to adjust for transfection efficiency.

All the transfections were performed at least 6 independent times and results are presented as the mean value for each sample. Values were determined at extract concentrations where the luciferase assays were in the linear range.

Analysis of Putative Transcription Factor Binding Sites. Analysis of putative transcription factors binding elements were done by computer analysis using the MatInspector v2.2 (Genomatix).

Nuclear extracts, electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from TCMK and MLE-15 cell lines by established procedures with minor modifications (Dignam *et al.* 1983. Nucleic Acids Res. 11:1475-1489). The salt concentration of the extraction buffer was 1.2 M KCl; the final concentration was adjusted to 300 mM KCl. 3T3 cell line nuclear extract was purchased from Geneka. EMSAs were performed using the Nushift Kit (Geneka) as recommended by the manufacturer. Five μg of nuclear extracts were incubated with 0.5ng ^{32}P end-labeled oligonucleotides probes for 20 minutes at 4°C with or without a 100-fold excess of cold competitor. For antibody-supershift-interference assays an anti-NF-κB p50 (Geneka) or an anti-NF-κB p52 (SantaCruz) rabbit polyclonal antibody was used by incubating nuclear extracts and antibodies for 20 min at 4°C. Bound and free probes were separated by nondenaturing electrophoresis in a 5% polyacrilamide gel.

Type II pneumocytes/Clara cells support preferential expression of the JSRV LTR. To determine the transcriptional activity of the JSRV LTR, transient transfection assays in different cell lines using a construct containing the firefly luciferase gene driven by the LTR of JSR21 (pJSRV21-luc) were performed. In each cell line, the relative activity of pJS21-luc was determined with respect to a promoter/enhancer plasmid (pGL3-control) containing a "neutral" promoter/enhancer (SV40, acted in many cell types) driving the same reporter gene. In all cases, co-transfections with a second reporter plasmid expressing a different reporter gene (*e.g.* pCMV-βgal expressing the β-galactosidase) also served to normalize transfection efficiencies between different experiments. Initial experiments were performed on

the MLE-15 line, which is a mouse cell line originating from lung tumors generated in transgenic mice harboring the SV40 large T antigen under the transcriptional control of the promoter/enhancer region from the human surfactant protein see (SP-C) gene (Wikenheiser, *et al.* 1993. Proc Natl Acad Sci U S A. 90:11029-11033). mtCC1-2 is a cell line derived from Clara cells from transgenic mice in a similar fashion but with the SV40 promoter under the transcriptional control of the CC10 promoter/enhancer (Magdaleno *et al.* 1997. Am J Physiol. 272:L1142-L1151). The activity of the JSRV LTR in lung cell lines vs. non lung cell lines such as those originating from testicular carcinoma, mammary carcinoma, mouse kidney cells, myoblasts, *etc.* (see table 1) were compared.

The relative activity of pGL3-control adjusted for transfection efficiency (by the co-transfected pCMV-βgal) was set to 100. The highest relative luciferase values for pJS21-luc were obtained in MLE-15, mtCCL-2 and MLE-12 with 429%, 109 %, and 16% of the activities of pGL3-control in the respective cell lines. Among the non-pulmonary cell lines, pJS21-luc showed the highest level of activity in 3T3 cells (11 percent). These results suggested that the JSRV LTR is preferentially expressed in cell lines derived from differentiated epithelial cells of the lungs.

Additional cell lines derived from sheep and humans were tested. Generally low activity was observed, even though the tested cell lines were derived from lung epithelial cells, including those that originated from human patients with bronchiolo-alveolar carcinoma (A549, H358 and H441 and the JS-7 cell line derived from a SPA tumor from a sheep. The fact that these BAC/SPA-derived cell lines showed relatively low expression of pJS21-luc may have been due to the fact that the cell lines generally have lost differentiation properties typical of BAC/SPA tumors and/or lung epithelial cells. The non-epithelial cell line that supported the highest levels of pJS21-luc activity was a sheep choroid plexus cell line (CP-PRI) obtained from the Moredun Research Institute, however, another sheep choroid plexus cell line obtained from the American type culture collection (CP-ATTC) supported expression of pJS21-luc very inefficiently.

To confirm the results in which pGL3-control (SV40 promoter/enhancer) was used as the reference neutral promoter/enhancer, the luciferase assays was repeated in selected murine and ovine cell lines using the Moloney murine leukemia virus (M-MuLV) LTR, the cytomegalovirus (CMV) immediate early promoter, or pGL3-basic (SV40 promoter but no enhancer) as "neutral" promoter/enhancer's. Transfection efficiency was adjusted with either pCMV-βgal (experiment series #1) or pRL-tk (experiment series #2) and the relative activity of the neutral promoter was set to 100.

In another set of experiments (#3 and 4), the relative activity of pJS21-luc was compared among the selected cell lines indicated above by comparing the straight ratios between the firefly luciferase values (induced by pJS21-luc transfected at 0.5 μg/well) and the *renilla* luciferase values used to normalize for transfection efficiency by using either pRL-tk or pRL-null (used at 50 ng/well).

Cell lines studied included MLE-15 and mtCC1-2 cells because they support high expression of pJS21-luc; 3T3 cells were the non-lung epithelial cells that supported the highest levels of pJS21-luc expression, while TCMK and St3 gave the lowest levels of expression. The ovine CP-MRI and CP-ATCC lines were also tested to compare murine and ovine cell lines. The results are shown in table 3.

In sets # 1 and 2, the relative activities of pJS21-luc in the different cell lines relative to the four "neutral" promoter/enhancer's are shown. Depending on the reference promoter/enhancer, there was variation in the relative strength of the JSRV LTR in the different cell lines. For instance, the activity of pJS21-luc in MLE-15 cells was approximately 200-fold greater than the SV40 promoter/enhancer, while it was 20-50-fold more active than the M-MuLV LTR in the same cell line. Nevertheless, among all the murine cell lines MLE-15 and mtCC1-2 consistently showed the highest activities regardless of the comparison "neutral" promoter/enhancer. In sets # 3 and 4, where pJS21-luc was co-transfected with *renilla* luciferase expression plasmids driven by either the HSV-tk or pRL-null the results were also consistent with the results of sets 1 and 2, in that the MLE-15 and mtCC1-2 cells consistently showed the highest levels of pJS21-luc activity. Overall, the results supported the implications that the two murine lung epithelial-derived cell

lines supported the highest transcriptional activity of the JSRV LTR.

Table 3 also shows expanded studies of the relative activities of pJS21-luc in the two sheep choroid plexus cell lines, CP-MRI and CP-ATTC. These two lines generally showed higher activities of pJS21-luc activity than the non-lung epithelial murine cell lines and in some cases also respect the lung murine cell lines, which might reflect higher activity of the JSRV LTR in ovine than murine cell lines. It was also noteworthy that overall the CP-MRI cells supported higher pJS21-luc activity than the CP-ATCC cells, consistent with the initial results. Similar experiments in which the concentrations of the co-transfected control plasmids were reduced, in order to rule out the possibility that promoter/enhancer elements on the co-transfected plasmids were titrating cellular transcription factors were performed. Essentially the same results as shown in table 3 were obtained.

The JSRV enhancers are particularly active in MLE-15 cells. To map transcriptional control elements in the JSRV LTR, a series of overlapping 5' truncations to two pJS21-luc were prepared. These truncations progressively eliminated four regions from the U3 region of the LTR: i) a distal region (-208 to -266); ii) a central distal region (-167 to -208); iii) a central proximal region (-51 to -167). The promoter proximal region of the LTR was considered to be from position 0 to -51. The deletions were then tested for transcriptional activity by transfection into various murine and ovine cell lines. The activities of the deletions are shown as folds of activation relative to pJS21(-37)-luc, a plasmid containing the JSRV LTR truncated and position -37. This plasmid would contain the putative basal promoter elements of the JSRV LTR, including the TATA box (position -23), as well as the R and U5 regions. The results showed that the central and distal elements were able to enhance transcriptional activity of the basal JSRV promoter in the murine MLE-15 and mtCC1-2 cell lines, with the strongest evidence for enhancer activity in MLE-15 cells. On the other hand, the other murine cell lines showed little evidence for enhancer activity for the JSRV LTR, with at most a two-fold difference between the full length pJS21-luc and the basal pJS21(-37)-luc. These results were very consistent with the results and table 3, in that the two lung epithelial cell lines that showed the highest level of transcriptional activity for the JSRV LTR also showed evidence for functional enhancer elements.

The results allowed localization of enhancer activity within the JSRV LTR for MLE-15 and mtCC1-2 cells. In particular, in MLE-15, approximately 40 percent of the enhancer activity was associated with elements between positions -51 and -240, while the remaining 60 percent was associated with the distal elements between -240 and -267. An internal deletion of the JSRV LTR (pJS21[Δ-209/-166]) was generated lacking the central distal elements, and had approximately 40 percent of activity in MLE-15 cells. This was consistent with the importance of the central distal elements of the JSRV LTR for enhancer activity in these cells.

When the sheep cell lines were tested with the truncation series, only the CP-MRI cells showed substantial evidence for enhancer activity. This was consistent with the higher JSRV LTR activity in those cells (table 3).

The contribution of elements downstream from the transcriptional start site (*e.g.*, R and U5) for the activity of the JSRV LTR were also tested. The U5 region appears to contain elements necessary for optimal expression, since deletion of the U5 region from pJS21-luc (construct pJS21U3R-luc) had 20-50 percent activity relative to pJS21-luc in all murine cell lines tested (MLE-15, mtCC1-2, 3T3 and TCMK). Addition of the first 63 nucleotides of U5 to this construct (pJS21[+63]-luc) fully or partially restored activity to the same levels of pJS21-luc activity in 3T3 and TCMK cells, but these nucleotides did not increase expression to the high levels of expression observed in MLE-15 or mtCC1-2 cells. Deletion of both R and U5 sequences from pJS21-luc reduced transcriptional activity to the background level given by pGL3-basic. Further studies will be required to elucidate the roles of R and U5 sequences in JSRV LTR-driven transcription.

Interaction between promoter and enhancer elements for optimal expression from the JSRV LTR. Both distal and central elements in the U3 region of the JSRV LTR contribute to optimal expression. It seemed that the most likely explanation was that the JSRV LTR contains enhancer elements that are particularly active in lung epithelial-derived cells. The JSRV enhancer elements (position -51 to -260) were examined to determine if it could confer cell-specificity to heterologous promoters. A series of luciferase reporter constructs were generated in which the JSRV enhancers were inserted in front of the basal SV40 or M-MuLV promoters. These constructs were tested for activity in MLE-15, 3T3 and TCMK cells. The activity of pJSp (or pSV-p or pMLV-p) after normalization for transfection efficiency with pCMV-βgal was taken as unit and compared to the activity of the various JS21 deletion mutants or the constructs containing heterologous promoters and heterologous enhancers. The JSRV enhancers were able to enhance expression from the SV40 and M-MuLV promoters in MLE-15 cells. Also, the JSRV enhancers were unable to enhance expression from the same promoters and TCMK cells, where there was little evidence for enhancer activity. In the case of 3T3 cells, results were somewhat unexpected, in that the JSRV enhancers were able to enhance expression of the SV40 and M-MuLV promoters; this enhancement was greater than the difference between the basal JSRV promoter and the full length JSRV LTR in these cells (pJSp vs pJSp+JSe, left panel).

Thus in 3T3 cells, the JSRV enhancers apparently are active, but they are more efficient at activating transcription from the heterologous SV40 and M-MuLV promoters than from the basal JSRV promoter. Similar results were obtained when a slightly larger portion of the JSRV LTR (-32 to -266) was placed in front of the basal SV40 promoter.

The fact that the JSRV enhancers combined with the JSRV basal promoter showed less enhancement in 3T3 cells than when they were placed in front of the heterologous promoters raised the possibility that the JSRV promoter may not be active in 3T3 (and/or TCMK) cells. To investigate this, a chimeric luciferase reporter construct in which the SV40 enhancers were placed in front of the basal JSRV promoter (pJSp+SVe) were prepared and its activity tested relative to the basal JSRV promoter and the full length JSRV LTR. The results indicated that the SV40 enhancers are able to activate expression of the basal JSRV promoter in all three cell lines. Thus the low expression of the native JSRV LTR in 3T3 cells (or TCMK cells) cannot be attributed to lack of basal promoter activity. These results suggest that high-level expression of the JSRV LTR in MLE-15 cells are the result (at least in part), not only to active enhancer and basal promoter elements, but appropriate interaction between these elements. Indeed, in MLE-15 cells the SV40 enhancers are less efficient at activating the basal JSRV promoter than are the JSRV enhancers, while in 3T3 and TCMK cells the converse is true.

The JSRV LTR response to cellular transcription factors involved in expression of lung surfactant proteins. In light of the demonstration that the JSRV LTR is preferentially active in lung epithelial-derived cell lines, the U3 region of the JSRV LTR was examined for potential binding sites for transcription factors known to be important for expression of genes in these cells. In particular, there are two putative HNF-3 binding sites; members of the HNF-3/forkhead family of nuclear transcription factors have been shown to be important in the regulation of surfactant gene expression (Margana and Boggaram. 1997. J Biol Chem. 272:3083-3090; Whitsett and Glasser. 1998. Biochim Biophys Acta. 1408:303-311; Hay and Crystal. 1997. Lung-specific gene expression, p. 277-304. In R. G. Crystal and J. B. West and E. R. Weibel and P. J. Barnes (ed.), The Lung: scientific foundations, 2nd ed, vol. 1.

Lippincott-Raven, Philadelphia). It has also been reported that other transcription factors such as NF-1, SP-1 and members of the octamer family cooperate with HNF-3/forkhead proteins in lung-specific expression, and these binding elements are also present in the JSRV LTR.

To test if putative binding elements in the JSRV LTR were important for expression, 3T3 cells were co-transfected with pJS21-luc along with expression plasmids for a series of transcription factors: TTF-1, HNF-3 alpha, HNF-3 beta, SP-1, HFH-8, and NF-1. The activation of JS21-luc by the various transcription factors was calculated by comparing the relative activity of pJS21-luc co-transfected with either a plasmid expressing the tested transcription factor driven by the CMV promoter or a plasmid with the CMV promoter alone. Transfection efficiency was normalized using pRL-null.

When the different amounts of the transcription factor expression plasmids were co-transfected with a standard amount of pJS21-luc, HNF-3α and HNF-3β luciferase expression was stimulated in a dose-dependent fashion. In contrast, HFH-8, another member of the HNF-3/forkhead family, did not activate expression of the JSRV LTR. It is interesting to note that HNF-3α and HNF-3β are expressed in type II pneumocytes and Clara cells, while HFH-8 expression is restricted to the epithelium and fibroblasts of the alveolar sac. It was also interesting that the JSRV LTR did not respond to co-transfection with the TTF-1 expression plasmid.

An NFκB binding site is important for expression of the JSRV LTR.

Approximately one-half of the enhancer activity of the JSRV LTR in MLE-15 and mtCC1-2 cells could be attributed to elements in the distal region (-239 to -266). This region contains an NFκB-like binding side with one mismatch (5'-GGGACGACC-3' (SEQ ID NO:__)) from the canonical NFκB consensus binding sequence (5'-GGGPuNNPyPyCC-3' (SEQ ID NO:____). To test if this binding side was important for the enhancer activity in the distal region of the JSRV LTR, a version of pJS21-luc was generated in which the NFκB-like site was mutated (pNFκBm-luc). The activity of pNFKBm-luc was compared to pJS21-luc in various cell lines and the relative

activity of pJS21-luc was set as 100.

Mutation of the NFκB-like site from the JSRV LTR reduced transcriptional activity in MLE-15 and mtCC1-2 cells while it did not affect the level of expression in the other murine cell lines. Thus these results support the idea that the NFκB-like elements is important for the high-level expression of the JSRV LTR in lung epithelial-derived cells, while it is not important for the low-level expression in non-lung epithelial cells. It was interesting that the only cell line in which mutation of the NFκB-like site showed a negative effect was the CP-MRI cells, which also show the highest expression of the JSRV LTR.

In view of the importance of the NFκB-like site for expression of the JSRV LTR in MLE-15 and mtCC1-2 cells, experiments were performed to test for the presence of nuclear factors that could bind to this sequence by electrophoretic mobility shift assays. Four major complexes of different mobilities could be detected in extracts from the MLE-15 cells when incubated with a labeled oligonucleotide containing the wild-type NFκB-like sequence, and these complexes could all be competed with excess wild-type oligonucleotide. When the same nuclear extracts were incubated with a mutant oligonucleotide corresponding to the mutation in pNFκBm-luc, two of the wild-type complexes were absent (the slowest and the most rapidly migrating ones) while two complexes were still detected. The complexes bound by the wild-type but not the mutant oligonucleotides seemed most likely to represent factors important in expression of the JSRV LTR. Experiments were performed to determine the presence of the NFκB site-binding proteins in 3T3 and TCMK cells, which do not support high-level expression of the JSRV LTR. Somewhat surprisingly, these cells also generated complexes that co-migrated with the complexes unique to wild-type oligonucleotide from MLE-15 cells. Thus the factor or factors that bind to the JSRV NFκB-like sequences may be ubiquitously expressed.

Antibodies to the p50 and p52 members of the NFκB proteins complex were used in attempts to supershift or inhibit complex formation in nuclear extracts from MLE 15 cells. However neither antibody showed inhibition or a supershift of any of

the complexes. Thus the proteins that bind to the NFκB-like site in the JSRV LTR may be previously unidentified NFκB-like proteins, or unrelated factors. It should be noted that the NFκB-like site also overlaps with an Ik-2-like binding site for Ikaros-related proteins. The Ikaros gene is expressed typically in hematopoietic cells, and there have not yet been reports of expression in differentiated lung cells.

These experiments demonstrate that JSRV long terminal repeats are preferentially activated in type II pneumocytes and Clara cells. This is supported by i) demonstrating that in transient transfection assays the JSRV LTR shows a preferential activation in mouse cell lines derived from type II pneumocytes (MLE-15) and Clara cells (mtCC1-2); ii) analysis of deletion mutants of pJS21-luc showed that the $JSRV_{21}$ enhancers are able to strongly activate the $JSRV_{21}$ proximal promoter in MLE-15; iii) the U3 of $JSRV_{21}$ contains putative enhancer binding motifs for transcription factors such as HNF-3, which have been involved in lung specific expression of the surfactant proteins and of the Clara cell protein CC10; iv) transactivation experiments demonstrated that HNF-3 is able to enhance the basal activity of pJS21-luc.

These data point to the LTRs as a determinant of the JSRV tropism for type II pneumocytes and Clara cells. The restriction in expression of JSRV in cell types other than type II pneumocytes and Clara cells, both *in vivo* and *in vitro*, is probably due to the lack of lung-specific transcription factors (or the presence of transcription repressors) which are necessary for the JSRV LTR to be activated. This also explains the difficulty to obtain a convenient tissue culture system for the propagation of JSRV. The most suitable substrate for JSRV replication *in vitro* would be type II pneumocytes and Clara cells isolated from sheep lungs.

Elements of the JSRV LTR located both upstream and downstream the TATA box seem to cooperate and be essential for optimal functionality and cell-specificity of the JSRV LTR. In promoter activation experiments we have shown that the JSRV enhancers (central and distal elements) are able to activate heterologous promoters (such as SV40 and MLV) but optimal (and cell-specific) activation is achieved only with the homologous JSRV promoter. It is also observed that deletion of the whole U5 or a portion of it reduces drastically pJS21-luc expression suggesting that

sequences downstream the transcriptional start site might have a role in JSRV transcription. However R and U5 are not by themselves capable of conferring tissue-specificity because a construct with the proximal elements of the JSRV U3, R and U5 (pJS21(-51)-luc) was activated by SV40 enhancers also in cell lines such as TCMK where the JSRV enhancers and the JSRV LTR showed to be poorly active. Further studies are necessary to establish the role of R and U5 in the JSRV LTR transcription. The R and U5 region might be necessary merely as a spacer for the JSRV promoter. On the other hand, R has been shown to be important for transcription not only in human and primate lentiviruses through the interaction between the tat protein and the TAR but also in other retroviruses such as murine leukemia viruses, mouse mammary tumor viruses, bovine leukemia viruses and the reticuloendotheliosis virus group member chicken syncitial virus.

Table 3. Comparison of the relative luciferase activity of pJS21-luc in selected cell lines by using different reference promoters and/or co-transfected plasmids to adjust for transfection efficiency.

	Set # 1				Set # 2				Set#3	Set#4
Cell Lines	SV	CMV	MLV	Basic	SV	CMV	MLV	Basic	JS21/ pRLTK	JS21/ pRLnull
MLE-15	223	9.7	22.8	11.2	187.2	52.1	50	11.6	77.2	56.1
mtCC1-2	51.3	15.2	22.7	12.4	90	93.6	76.3	5.6	62.9	29.9
3T3	5.9	2.4	7.8	4.5	5.9	46	24	3.9	16.3	5.4
TCMK	1	1	1	1	1	1	1	1	1	1
ST3	2.9	3.1	14.3	0.8	6.5	15	34.7	1	11.6	6.8
CP-MRI	7.1	6.6	4	13.2	29.2	46.2	34	9.4	26.8*	76.5*
CP-ATCC	4.3	4.3	3.2	6.1	6.6	10.4	9.9	5.4	24	13

EXAMPLE 3

CHARACTERIZATION OF JAAGSIEKTE SHEEP RETROVIRUS-RELATED ENDOGENOUS RETROVIRUSES OF SHEEP

Molecular cloning. The construction of a high molecular weight genomic DNA lambda phage library derived from an OPC lung tumour DNA is described above. The library was divided into 15 sub-library and each sub-library was independently amplified. Aliquots of the 15 sublibraries were screened for the presence of exogenous JSRV proviruses by using a JSRV U3-specific hemi-nested PCR (U3 hn-PCR). The sub-libraries negative for exogenous JSRV were further screened for the presence of *enJSRVs*. In particular sublibrary #5 and #6 were plated onto bacterial agar plates and subjected to hybridization of plaque lifts with two ^{32}P-labelled probes on replica filters: a *gag*-specific probe and an *env*-specific probe. Under the hybridization conditions used, these probes hybridized with both endogenous and exogenous JSRV sequences. Primary plaques positive for both probes were picked and further purified by dilution and plating for isolated plaques on bacterial lawns, followed by hybridization with both *gag* and *env* probes. The presence of exogenous JSRV was ruled out by LTR exogenous-specific PCR and by the lack of the exogenous specific *ScaI* restriction site in *gag*. Three recombinant phages carrying three distinct *enJSRVs* loci were subcloned into pBlueScript (Stratagene) to give penJS56A1, penJS59A1 and penJS51F6. Both strands of the three clones were completely sequenced on an ABI Prism 310 Genetic Analyser (Perkin-Elmer), using a BigDye Terminator DNA cycle sequencing kit (PE Applied Biosystems) as recommended by the manufacturer.

Computer analysis of sequence data. Sequences were analyzed using DNASTAR 1.59 software package (DNASTAR, Inc.) and DNA Strider 1.2. Sequences alignments were performed using ClustalW 1.8. Phylogenetic analysis was performed calculating the genetic distances between sequence pairs by the DNADIST program in PHYLIP version 3.5. Neighbor-joining trees were estimated by NEIGHBOR program and a bootstrap analysis using 1,000 bootstrap replications.

Plasmids. Plasmid pCMV2JS21 is a construct derived from the $JSRV_{21}$ infectious molecular clone where the viral genes are under the control of the cytomegalovirus immediate-early promoter as describe above. Plasmid pCMV2en56A1 was derived by replacing the 5' LTR of pen56A1 with the CMV, R and U5 of $pCMV2JS_{21}$ by standard molecular techniques. Chimeric constructs

between pCMV2JS$_{21}$ and pCMV2en56A1 were obtained taking advantage of the *HpaI* and *BamHI* restriction sites present respectively in *gag* (position 1274 of JSRV$_{21}$) and at the end of *pol* (position 5265 of JSRV$_{21}$, 135bp before the end of the *pol* reading frame and 56 bp before the start codon of *env*) in both constructs. Plasmid pGPxEe has *gag* and *pol* of pCMV2JS$_{21}$ and *env* from pCMV2en56A1 while plasmid pGPeEx has the *gag* and *pol* from pCMV2en56A1 and the *env* from pCMV2JS$_{21}$. Plasmid pGePEx has the majority of *gag* from pCMVen56A1 and *pol* and *env* from pCMV2JS$_{21}$ while pGxPEe on the contrary has the first two-thirds of *gag* from the exogenous pCMV2JS$_{21}$ and the rest of the genome from pCMVen56A1.

The LTR of penJS5F16, penJS56A1 and penJS59A1 were cloned into pGL3-basic (Promega) by standard PCR cloning techniques. The resulting plasmids (pen5F16-luc, pen56A1-luc and pen59A1-luc) have the firefly luciferase gene under the control of the various endogenous LTR. pJS21-luc has instead the LTR of JSRV$_{21}$ which drives the firefly luciferase gene. pRL-null (Promega), a promoterless plasmid with the *renilla* luciferase gene was used to adjust the transfection efficiency in the luciferase assays described below.
Plasmids pCMV-HNF3α and pCMV-HNF3β, expressing the hepatocyte nuclear factor (HNF) 3α and β (provided by R.H. Costa, University of Illinois, Chicago) were used in transactivation experiments.

Cell cultures. MLE-15 cell line, a mouse type II pneumocytes-derived cell line (provided by J. Whitsett), was grown in RPMI 1640 (Gibco BRL), 2% FBS, 0.5 % ITS (Sigma) modified with the addition of 5mg/l transferrin, 10 mM Hepes, 1X10^{-8} M β-estradiol and 1X10^{-8} M Hydrocortisone. Human 293T cell line, mtCC1-2, derived from mouse Clara cells (provided by F. DeMayo), and NIH-3T3 (ATCC#CCL-92) were grown in DMEM (ATCC) and 10% FBS. TCMK (ATCC#CCL-139) cell line (derived from mouse kidney) were grown in DMEM (ATCC), 1X non-essential amino acids (Cellgro) and 10% FBS. The LE cell line (provided by T. Spencer) derives from sheep endometrium epithelium and was grown in F12-K (Gibco BRL) 10% FBS. All the cell lines were grown in an incubator at 37°C with 5% CO_2.

Transient transfections and luciferase assays. Transient transfections were performed on 2-4 X 10^5 cells plated on six well plates (Falcon) approximately 24 hours prior to transfection. For each well, 500ng of reporter plasmid and 50ng of pRL-null were used with 6 µl of Fugene (Boehringer) as recommended by the manufacturers. Experiments were done in 12 replicates. Cells were lysed 48 h after transfection using the dual luciferase reporter system (Promega) protocol in a TD 20/20 luminometer (Turner Design) as recommended by the manufacturer. The values of the various endogenous LTR reporters were compared to the activity of pJS21-luc which was taken as 100%.

For the transactivation experiments, 200ng of pJS21-luc, 1 to 200ng of transactivating plasmid (or control plasmid containing the same promoter as the transactivating plasmid) and 50 ng of pRL-null have been used in NIH-3T3 cells. The activation of JS21-luc by HNF-3 expression plasmids was calculated by comparing the relative activity of pJS21-luc co-transfected with either pCMVHNF-3α (or pCMVHNF-3β) or a plasmid with the CMV promoter alone. Transfection efficiency was normalized as above using pRL-null.
For the production of viral particles, 293T cells were transfected with pCMV2JS21 or pCMV2en56A1 (or the various chimeras), and viral particles were collected from concentrated supernatants as described above.

Western blotting. Western blotting of concentrated 293T supernatants for the detection of JSRV major capsid protein were performed as described above.

Tissue Samples. Tissue samples used for *in situ* hybridizations were collected during the necroscopy of a healthy sheep. Tissues analyzed were lungs, liver, kidney, spleen, uterus, intestine/jejunal Peyer's patches, mediastinal lymph nodes, pre-crural lymph nodes, jejunal lymph nodes. Samples were fixed in 10% neutral buffered formalin, processed routinely in an automatic tissue processor, embedded in paraffin wax and sectioned (5-7 µm).

In situ hybridization. Deparaffinized, rehydrated, and deproteinated tissue sections were hybridized with radiolabeled antisense or sense cRNA probe generated

from linearized plasmid template (DD54) by *in vitro* transcription with [α-^{35}S]UTP (3000 Ci/nmol; Amersham-Pharmacia). DD54 contains 436 bp from the *env* region of *enJSRVs* and is 96 to 98% identical to enJS56A1 and enJS5F16. Autoradiographs of slides were prepared using Kodak BioMax MR film exposed for 16 h. Autoradiography was performed using Kodak NTB-2 liquid photographic emulsion. Slides were kept at 4 °C for 1 week, developed in Kodak D-19 developer, counterstained with Harris' modified hematoxylin in acetic acid (Fisher), dehydrated through a graded series of alcohol to xylene, and cover-slipped. Photomicrographs were taken under brightfield and darkfield illumination using a Carl Zeiss Axioplan2 photomicroscope fitted with a Hamamatsu chilled 3CCD color camera.

Nucleotide sequences accession numbers. Sequences of pen56A1, pen5F16 and pen59A1 have been deposited in GenBank with the following accession numbers AF153615, AF136224 and AF136225 .

From the cloning procedure described above three full length endogenous proviral loci termed enJS56A1, enJS5F16 and enJS59A1 were obtained. The length of the proviruses was 6915bp for enJS5F16, 7939bp for enJS56A1 and 6695bp for enJS59A1. The genomic structure of the three loci is schematically presented in Fig. 4.

All three endogenous loci have an upstream and a downstream LTR, hallmark of complete proviruses. In the U3 region of the LTR there were major differences respect to the exogenous JSRV. The LTR of the endogenous loci are also longer with respect to the exogenous JSRV and ENTV and the difference in length is all given by the U3 region. The length of U3 of the endogenous loci varies between 301 (enJS59A1) to 319 bp (enJS56A1 and enJS5F16) while for the exogenous JSRV is 272 and only 250bp for ENTV. The U3 region of enJSRV5F16 and enJSRV56A1 were 98% identical between each other and 85% respect enJSRV59A1. The endogenous loci showed approximately 74% identity respect the $JSRV_{21}$ U3 while R and U5 were highly homologous among the endogenous loci and respect $JSRV_{21}$. The upstream and downstream LTRs of enJSRV5F16 are identical while those of enJSRV56A1 and enJSRV59A1 display two and four base changes.

All the three endogenous loci have a conserved $tRNA^{lys}_{1,2}$ primer binding site (PBS) which is the same used by the exogenous JSRV and ENTV. The *gag* gene shows an intact open reading frame in enJSRV56A1 and enJSRV5F16 while a single bp insertion creates a frameshift with a downstream termination codon at position 820 of the enJSRV59A1 provirus. The whole Gag is 98.2% identical in the endogenous clones. The Gag polyprotein is highly conserved between the endogenous and exogenous viruses (94 to 95% identity) with the exception of a short region corresponding to the matrix (MA) of $JSRV_{21}$ between nt 624 and 661 that shows a proline-rich motif where there is no real homology (Fig. 5) between the endogenous clones and the exogenous JSRV. Interestingly this region shows also some polymorphism between JSRV and ENTV where there is only 50% identity at the amino acid level compared to the 95.8% identity for the whole Gag. We termed this region VRA (variable region A) for the type D retroviruses of sheep. Downstream the VRA (50 aa residues) there is another region that shows polymorphism between endogenous and exogenous viruses; we termed this region VRB. It is interesting to note that ENTV in VRB, seem to be more closely related to the endogenous loci than to the exogenous JSRV.

The *pro* region shows an uninterrupted open reading frame and it is highly homologous among all the three endogenous clones. A very high homology it is shown also between the endogenous clones and the exogenous $JSRV_{21}$ (95 to 99.7% identity). The dUTPase motifs found in the 5' half of the *pro* gene of JSRV are conserved in the endogenous loci.

pol shows major defects in enJS5F16 and enJS59A1: in enJS5F16 there are two large deletions of 154 and 872 bp while a point mutation in enJS59A1 creates a stop codon (position 4071 of the provirus sequence).

In enJS56A1 there is a two bp deletion respect the exogenous JSRV at the 3' end of the gene (corresponding at the integrase region) that results in a theoretical polypeptide 14 aa shorter respect the exogenous product and with the last 33 amino acid with no homology with the exogenous amino acid sequence. The Pol

polyproteins of enJS56A1 and $JSRV_{21}$ are 97.8% identical (this value exclude the last 33aa of the endogenous locus).

The *orf-x* region which overlap *pol* is an uninterrupted open reading frame in enJSRV59A1. In enJSRV5F16 there is an ample truncation as a consequence of the deletion in *pol* while in enJSRV56A1 there is a stop codon 39bp before the stop codon of the $JSRV_{21}$ *orf-x*.

The *env* gene is deleted in enJSRV59A1 while it is a fully open reading frame in enJS5F16 and enJS56A1. This region is 98% identical at the aminoacidic level between the two endogenous loci and around 92% identical between endogenous and exogenous $JSRV_{21}$ sequences. In the last 67 aa of Env (in the TM region) there is another region of high divergency between endogenous and exogenous sequences (57 to 59% aa identity). This region has been shown to be also high variable between JSRV type I (composed of isolates from the African continent) and JSRV type 2 (from the UK and USA) sequences. This region was termed VRC. VRC shows to be highly variable also between exogenous JSRV and ENTV sequences (Fig. 6).

Of the three endogenous loci cloned enJS56A1 has uninterrupted open reading frames in all the structural genes. The only apparent defects of enJS56A1 are a premature stop codon in the *orf-x* and a two bp deletion respect the exogenous JSRV at the 3' end of the *pol* gene (corresponding to the integrase region) that results in a theoretical polypeptide 14 aa shorter respect the exogenous product and with the last 33 amino acids with no homology with the exogenous amino acid sequence. The *orf-x* is not necessary for viral particle formation and infectivity *in vitro*.

In order to test whether the enJS56A1 has the potential to express viral particles a construct was generated where transcription is driven by the cytomegalovirus immediate-early promoter (termed pCMV2en56A1) (Fig.7). In pCMV2en56A1 the upstream LTR of penJS56A1 has been replaced with the CMV, the R and U5 regions from $pCMV2JS_{21}$. $pCMV2JS_{21}$ is a derivative of $pJSRV_{21}$ where the CMV immediate-early promoter drives JSRV transcription and it has been a useful tool to produce JSRV infectious virus *in vitro* by transiently transfecting 293T

cells and collecting viral particles in the resulting supernatant. 293T cells were transfected in parallel with pCMV2JS21 and with pCMV2en56A1 and their supernatants harvested at 24, 48 and 72h post-transfection. The resultant pools were ultracentrifuged over a double cushion of glycerol and the pelletts subjected to SDS-PAGE/ western blotting using a rabbit antiserum towards the major capsid protein of JSRV. A band of 26 kDa was observed in the concentrated supernatants of pCMV2JS$_{21}$ transfected cells, as expected, but no band was detected in the pCMV2en56A1 supernatants. This indicates that enJS56A1 is unable to form virus particles. To localize the region/s responsible for this defect chimeric constructs were made between pCMV2JS$_{21}$ and pCMV2en56A1 (Fig.7) as described herein. pGPxEe has *gag* and the majority of *pol* from the exogenous JSRV$_{21}$ and the 3' 180 bp of *pol* and the entire *env* from enJS56A1; pGPeEx is the opposite chimera with *gag* and the majority of *pol* from the endogenous locus and *env* from JSRV$_{21}$. pGePEx has instead the first 2/3 of *gag* from the endogenous enJS56A1 and the rest of the genome from pCMV2JS21 and pGxPEe is the opposite chimera with *gag* endogenous and *pol* and *env* endogenous. pGPxEe was able to produce viral particles. A western blot of 300-fold-concentrated supernatant from 293T transiently transfected with the constructs showed a positive signal from lung fluid, pCMV2JS$_{21}$ and pGPxEe suggesting that the defect for packaging for enJS56A1 is contained upstream the *HpaI* site in *gag*. The 26 kDa protein is indicated; the defect for viral packaging is not therefore due to the slippage of the 3' portion of the *pol* open reading frame that is after the *BamHI* site used to make this chimera. Conversely neither pGPeEx or pGePEx were able to produce viral particles while pGxPEe did produce viral particles. The defect for packaging is therefore localized in the first two/thirds of *gag*, upstream the *HpaI* site (position 1274 of JSRV$_{21}$); interestingly this is where the two variable regions, VRA and VRB, are contained. However any single amino acid change outside this regions or polymorphism in the untranslated *gag* (Fig. 8) might also determine the packaging defect of enJS56A1.

Phylogenetic analysis and evolution. Unrooted neighbor-joining phylogenetic trees to assess the phylogenetic relationship between the three endogenous loci that were cloned and other known sequences of endogenous and exogenous type D retroviruses of sheep were generated. A tree for the U3 region, one for *env* and one for

gag and *pol* (Fig 9a to c) were generated. In each tree it is possible to distinguish three major branches: one for the endogenous loci, one for the exogenous ENTV and one for the exogenous JSRV sequences confirming previous analysis performed with limited *gag* sequences. The exogenous JSRV are further divided into two branches corresponding to sequences derived from the UK or from Africa and the USA. In all the generated trees the enJS59A1 branches apart from the other two endogenous loci cloned in this study and from most of the previous endogenous sequences generated by PCR-cloning.

The endogenous type D retroviruses loci seem to be quite young from the evolutionary point of view. An estimate of the time of integration in the sheep germline of these elements can be made by taking into account the variability between 5' and 3' LTR of a single locus. The intragenomic changes reflect changes that have accumulated since the time of integration in the sheep germline since it can be safely assumed that these LTRs were identical at the time of integration and were then subjected to non-coding regions or pseudogenes mutation rates. In other words, intragenomic variability of the LTRs can be used as a molecular clock to estimate time of integration. Thus, the integration events of enJSRV56A1 and enJSRV59A1 happened approximately 0.9 and 1.8 millions years ago using the average value of 4.85×10^{-9} substitutions per nucleotide site per year relative to pseudogenes. enJS5F16 might have integrated less than 500000 years ago. These numbers are subject to a margin of error and do not take into account the possibility of gene conversion.

From the constructed trees (Fig. ___), the endogenous loci can be divided in at least two phylogenetic groups: *enJSRV-A*, *enJSRV-B*. Another one or two groups might arise (*e.g.*, proviruses where the sequences locus 5 and 6 were derived might form a group separate from *enJSRV-A*) but complete proviral sequences need to be obtained in order to fully classify these elements.

Expression of *enJSRVs in vivo*. To evaluate the expression of *enJSRVs in vivo* *in situ* hybridization was performed in a panel of tissues collected from healthy sheep. The DD54 probe that contains 436bp of the *env* gene and it is 96-98% identical to enJS56A1 and enJS5F16 *env* was used. DD54 was derived in a study aimed to isolate

messengers differentially expressed in the uterine epithelium. Indeed the luminal epithelium and the glandular epithelium of the uterus was where a very strong hybridization signal was detected. Cells in the lamina propria of the gut also showed some positivity and some positive signal above background was detected in the bronchiolar epithelium in the lungs. The alveolar epithelium did not show signal above background. Very weak signal above background or no signal above background was detected in the liver, kidney, spleen, tonsils and peripheral lymph nodes.

As discussed above, the long terminal repeat of JSRV are preferentially expressed in cell lines derived from the epithelial cells of the lungs (type II pneumocytes and Clara cells). To assess whether the pulmonary tropism is common between the exogenous and endogenous viruses reporter assays were performed with luciferase expressing constructs driven by the $JSRV_{21}$ LTR (pJS21-luc) or by the LTR of each one of the three endogenous loci (enJS56A1-luc, enJS5F16-luc and enJS59A1-luc). Results are expressed as % luciferase activity respect pJS21-luc (=100%) after adjustment for transfection efficiency by measuring the *renilla* luciferase values induced by the co-transfected pRL-null.

These experiments were performed in five different cell lines: MLE-15 (a mouse cell line derived from type II pneumocytes), mtCC1-2 (derived from mouse Clara cells), TCMK (derived from mouse kidney), NIH-3T3 (mouse embryo fibroblasts) and LE cells (a sheep cell line derived from the endometrial epithelium). JS21-luc had the highest relative luciferase activity in MLE-15 and mtCC1-2; NIH-3T3 had an intermediate level of JS21-luc expression while TCMK had low expression of pJS21-luc, as described above. The LE cells were chosen because of a previously reported study where *enJSRVs* transcripts *in vivo* have been shown in the epithelium of the endometrium. As it is schematically shown in Fig. 10, the endogenous LTRs had a much lower luciferase activity respect pJS21-luc in the lung derived cell lines MLE-15 and mtCC1-2. The activity of the endogenous LTRs ranged from 7 to 11% (respect pJS21-luc) in MLE-15 cells and from 17 to 24% in mtCC1-2. However in TCMK, 3T3 and LE cells the activity of the endogenous LTR clones

were in the great majority of cases comparable (45% to 115%) with the activity of pJS21-luc.

This result suggest that the pulmonary tropism of JSRV has been probably acquired during the evolution and that the JSRV-like exogenous virus that gave origin to the *enJSRVs* elements did not show the tropism for the differentiated epithelial cells of the lungs that it is shown by the contemporary JSRV.

In addition, the endogenous clones were transactivated by HNF-3. HNF-3 which is a transcription factor which has been shown to play a major role in the lung-specific transcription. JSRV LTR have two hypothetical HNF-3 responsive elements and are transactivated in 3T3 cells by HNF-3. In the transactivation experiments shown in Fig.11, none of the endogenous LTR clones responded to HNF-3α or HNF-3β while the exogenous JSRV was activated by both HNF-3α and β as expected.

Three loci of type D endogenous retroviruses of sheep (*enJSRVs*) were clones and showed their proviral structure, phylogeny and pattern of expression. All the three loci have fully open reading frames for at least one or more of the structural genes. In particular, enJS56A1 is an apparently full length provirus with open reading frames for *gag*, *pol* and *env*. enJS56A1 is however unable to make viral particles and by the construction of viral chimeras between the exogenous $JSRV_{21}$ and enJS56A1 we were able to identify the first two/third of *gag* of enJS56A1 as the main region where the defect for particle formation lies. For the first time two short regions in *gag* (VRA and VRB) where major differences between endogenous and exogenous type-D retroviruses of sheep are localized were identified. In VRA, in particular, is contained a proline-rich region in both JSRV and ENTV that it is absent in the endogenous loci. A third region that is divergent between exogenous and endogenous sequences is localized in the carboxy terminal portion of the transmembrane (TM) protein (that we termed VRC). Interestingly, in these variable regions there is also an high polymorphism between JSRV and ENTV. Future studies might need to further investigate VRA, VRB and VRC to evaluate if they influence the pathogenicity and/or tropism of the oncogenic exogenous viruses.

With the exception of these three variable regions, the endogenous loci are remarkable similar to their exogenous counterparts in all the genes; a strong polymorphism is instead localized in the U3 where the retroviral promoter and enhancers are located. The JSRV LTR has been shown to be a main determinant of viral tropism for the epithelial cells of the lungs, as described above. By *in situ* hybridization, it was shown that the strongest expression of *enJSRVs* seem to be localized in the luminal epithelium and in the glandular epithelium of the uterus. High expression of these elements along the genital tract might have been necessary for integration in the germ cells allowing in this way the generation of endogenous viruses. Weaker expression was detected in the lamina propria the gut and in the bronchial epithelium of the lung but no signal above background was detected in the alveolar epithelium.

By reporter assays it was shown that the LTR of the three cloned endogenous loci do not share the same lung-tropism of the exogenous JSRV. This suggests that JSRV has developed its lung tropism during the evolution as an exogenous virus. In other words the exogenous virus that integrated into the sheep germline to give origin to the endogenous loci did not show strong pulmonary tropism. This hypothesis is reinforced by the fact that the LTR of the various *enJSRVs*, on the contrary of the JSRV LTR, are not transactivated by HNF-3, a transcription factor involved in lung-specific gene expression.

Based on the analysis of the variability between 5' and 3' LTR of the same locus is estimated that the time of integration for enJS56A1 and enJS59A1 was between 0.9 and 1.8 million years ago while enJS5F16 might have integrated even less than 500000 years ago. This is of course an estimation. Hecht *et al* showed that sheep (plus the wild members of the genus *Ovis*) and goats (plus the wild members of the genus *Capra*) have both approximately 20 copies of type D-related endogenous retroviruses; the restriction profile of these elements is different between the two gene but it is similar among members of the same genus. This imply the most of the *enJSRVs* loci have been acquired after the divergence between sheep and goats that occurred 4 to 10 million years ago. From these data it is suggested that the *enJSRVs* are rather young from the evolutionary point of view and they can be considered as

"modern" endogenous retroviruses; the existence of the closely related exogenous JSRV and ENTV is indeed another clue that these elements are evolutionarily young.

While the methods and compositions described above are typical of those that can be used to carry out certain aspects of the invention, other procedures known to those skilled in the art can also be used.

In addition, numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described. It is to be understood that, while the invention has been described with reference to the above detailed description, the foregoing description is intended to illustrate, but not to limit, the scope of the invention. Other aspects, advantages, and modifications of the invention are within the scope of the following claims. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

What is claimed is:

1. An isolated replication competent infectious Jaagsiekte sheep retrovirus (JSRV).

2. The isolated retrovirus of claim 1, wherein the retrovirus comprises:
a JSRV GAG protein;
a JSRV POL protein;
a JSRV ENV protein;
a JSRV genome comprising Long-Terminal Repeat (LTR) sequences at the 5' and 3' end of the retroviral genome, wherein the LTR is active in pulmonary epithelial cells,
a polynucleotide sequence encoding JSRV GAG protein, JSRV POL protein, and JSRV ENV protein; and
cis-acting nucleic acid sequences necessary for reverse transcription, packaging and integration in a target cell.

3. The isolated retrovirus of claim 1, having a genomic sequence as set forth in GenBank accession no. AF105220.

4. A recombinant replication competent Jaagsiekte sheep retrovirus (JSRV) comprising:
a JSRV GAG protein;
a JSRV POL protein;
a JSRV ENV protein;
a JSRV genome comprising Long-Terminal Repeat (LTR) sequences at the 5' and 3' end of the retroviral genome, wherein the LTR is active in pulmonary epithelial cells,
a heterologous nucleic acid sequence operably linked to a regulatory nucleic acid sequence; and
cis-acting nucleic acid sequences necessary for reverse transcription, packaging and integration in a target cell.

5. The retrovirus of claim 4, wherein the ENV protein further comprises a target-specific ligand sequence.

6. The retrovirus of claim 5, wherein the targeting specific ligand sequence is an antibody, receptor, or ligand.

7. The retrovirus of claim 5, wherein the target cell is a pulmonary cell.

8. The retrovirus of claim 5 wherein the target cell is a cell having a cell proliferative disorder.

9. The retrovirus of claim 8, wherein the cell proliferative disorder is selected from the group consisting of lung cancer, colon-rectum cancer, breast cancer, prostate cancer, urinary tract cancer, uterine cancer lymphoma, oral cancer, pancreatic cancer, leukemia, melanoma, stomach cancer and ovarian cancer.

10. The retrovirus of claim 5, wherein the heterologous polynucleotide sequence is a suicide gene.

11. The retrovirus of claim 10, wherein the suicide gene is a thymidine kinase.

12. The retrovirus of claim 5, wherein the heterologous sequence is a marker gene.

13. An isolated Jaagsiekte sheep retrovirus (JSRV) genome, comprising:
a polynucleotide as set forth in GenBank accession no. AF105220.

14. The isolated JSRV of claim 13 contained in an expression vector.

15. The isolated JSRV of claim 14, wherein the vector is a plasmid.

16. The isolated JSRV of claim 14, wherein the vector contains a regulatory sequence in operable association with JSRV genomic sequence.

17. The isolated JSRV of claim 16, wherein the regulatory sequence is a CMV early promoter sequence.

18. An isolated polynucleotide comprising the nucleic acid sequence as set forth in GenBank accession number AF105220, sequences complementary thereto and variants and fragments thereof.

19. The isolated polynucleotide sequence of claim 18, wherein T can be U and sequences complementary thereto.

20. An expression vector having in operable association the polynucleotide of claim 18.

21. A host cell transformed with the expression vector of claim 20.

22. A method for producing an infectious Jaagsiekte sheep retrovirus (JSRV), comprising:

transfecting a host cell with the vector of claim 20;

culturing the host cell under sufficient conditions and for sufficient time to allow expression of the plasmid to produce JSRV viral particles; and

obtaining the JSRV viral particles.

23. The method of claim 22, wherein the host cell is a pulmonary epithelial cell.

24. The method of claim 22, wherein the host cell is selected from the group consisting of a human 293T cell, a mtCCl-1 cell, and an MLE-15 cell.

25. A method of treating a subject having a cell proliferative disorder, comprising:
contacting the subject with a retroviral vector, comprising,
a JSRV GAG protein;
a JSRV POL protein;
a JSRV ENV protein;
a JSRV genome comprising Long-Terminal Repeat (LTR) sequences at the 5' and 3' end of the retroviral genome, wherein the LTR is active in pulmonary epithelial cells,
a heterologous nucleic acid sequence operably linked to a regulatory nucleic acid sequence; and
cis-acting nucleic acid sequences necessary for reverse transcription, packaging and integration in a target cell.

26. The method of claim 25, wherein the subject is a mammal.

27. The method of claim 26, wherein the mammal is a human.

28. The method of claim 25, wherein the contacting is by *in vivo* administration of the retrovirus.

29. The method of claim 28, wherein the *in vivo* administration is by systemic, local, or topical administration.

30. The method of claim 25, wherein the contacting is by *ex vivo* administration of the retrovirus.

31. The method of claim 25, wherein the ENV protein further comprises a target-specific ligand sequence.

32. The method of claim 31, wherein the targeting specific ligand sequence is an antibody, receptor, or ligand.

33. The method of claim 25, wherein the target cell is a cell having a cell proliferative disorder.

34. The method of claim 33, wherein the cell proliferative disorder is selected from the group consisting of lung cancer, colon-rectum cancer, breast cancer, prostate cancer, urinary tract cancer, uterine cancer lymphoma, oral cancer, pancreatic cancer, leukemia, melanoma, stomach cancer and ovarian cancer.

35. The method of claim 25, wherein the heterologous polynucleotide sequence is a suicide gene.

36. The method of claim 25, wherein the suicide gene is a thymidine kinase.

37. A pharmaceutical composition useful for inducing an immune response to Jaagsiekte sheep retrovirus (JSRV) in an subject comprising an immunogenically effective amount of a JSRV or JSRV polypeptide in a pharmaceutically acceptable carrier.

38. The pharmaceutical composition of claim 37, wherein the JSRV is a non-infectious JSRV.

39. The pharmaceutical composition of claim 37, wherein the JSRV is a heat inactivated JSRV.

40. The pharmaceutical composition of claim 37, wherein the JSRV polypeptide is an env polypeptide.

41. The pharmaceutical composition of claim 37, wherein the pharmaceutically acceptable carrier contains an adjuvant.

42. A method of inducing an immune response to a JSRV or JSRV polypeptide in a subject, comprising immunizing the animal with the composition of claim 37.

43. An antibody which specifically binds to the replication competent infectious Jaagsiekte sheep retrovirus (JSRV) of claim 1.

44. The antibody of claim 43, wherein the antibody is a monoclonal antibody.

45. A method for inhibiting the binding of a JSRV to a cell comprising contacting the JSRV with an anti- JSRV-antibody.

46. The method of claim 45, wherein the anti-JSRV antibody binds to a JSRV envelop protein.

47. The method of claim 45, wherein the contacting is *in vivo*.

48. The method of claim 45, wherein the contacting is *in vitro*.

49. The method of claim 45, wherein the antibody is formulated in a pharmaceutically acceptable carrier.

50. A method for identifying a compound which binds to a Jaagsiekte sheep retrovirus (JSRV) comprising:

a) incubating components comprising the compound and the JSRV under conditions sufficient to allow the components to interact; and

b) measuring the binding or effect of binding of the compound to the JSRV.

51. The method of claim 50, wherein the compound is a peptide.

52. The method of claim 50, wherein the compound is a peptidomimetic.

53. The method of claim 50, wherein measuring the ability of the compound to bind to the JSRV is by detection of a infectivity of the JSRV.

54. A method for inhibiting the expression of Jaagsiekte sheep retrovirus (JSRV) in a cell comprising contacting the cell with an inhibiting effective amount of an antisense oligonucleotide that binds to a segment of an mRNA transcribed from the JSRV genome whereby the binding of the antisense to the mRNA segment inhibits JSRV gene expression.

55. A recombinant retroviral vector, comprising:
a GAG protein;
a POL protein;
a ENV protein;
a polynucleotide sequence comprising jaagsiekte sheep retrovirus Long-Terminal Repeat (LTR) sequences at the 5' and 3' end of the polynucleotide sequence, wherein the LTR is active in pulmonary epithelial cells, a gag nucleic acid sequence, a pol nucleic acid sequence and an env nucleic acid sequence; and
cis-acting nucleic acid sequences necessary for reverse transcription, packaging and integration in a target cell.

56. The retroviral vector of claim 55, further comprising a heterologous sequence.

57. The retroviral vector of claim 55, wherein the gag, pol and env sequence are letiviral gag pol and env sequences.

58. The retroviral vector of claim 55, wherein the ENV protein is a JSRV ENV protein.

59. A method of driving lung-specific expression of a heterologous polynucleotide sequence comprising contacting a lung cell with a vector comprising a jaagsiekte sheep retrovirus long terminal repeat sequence (LTR) operably linked to the heterologous polynucleotide sequence.

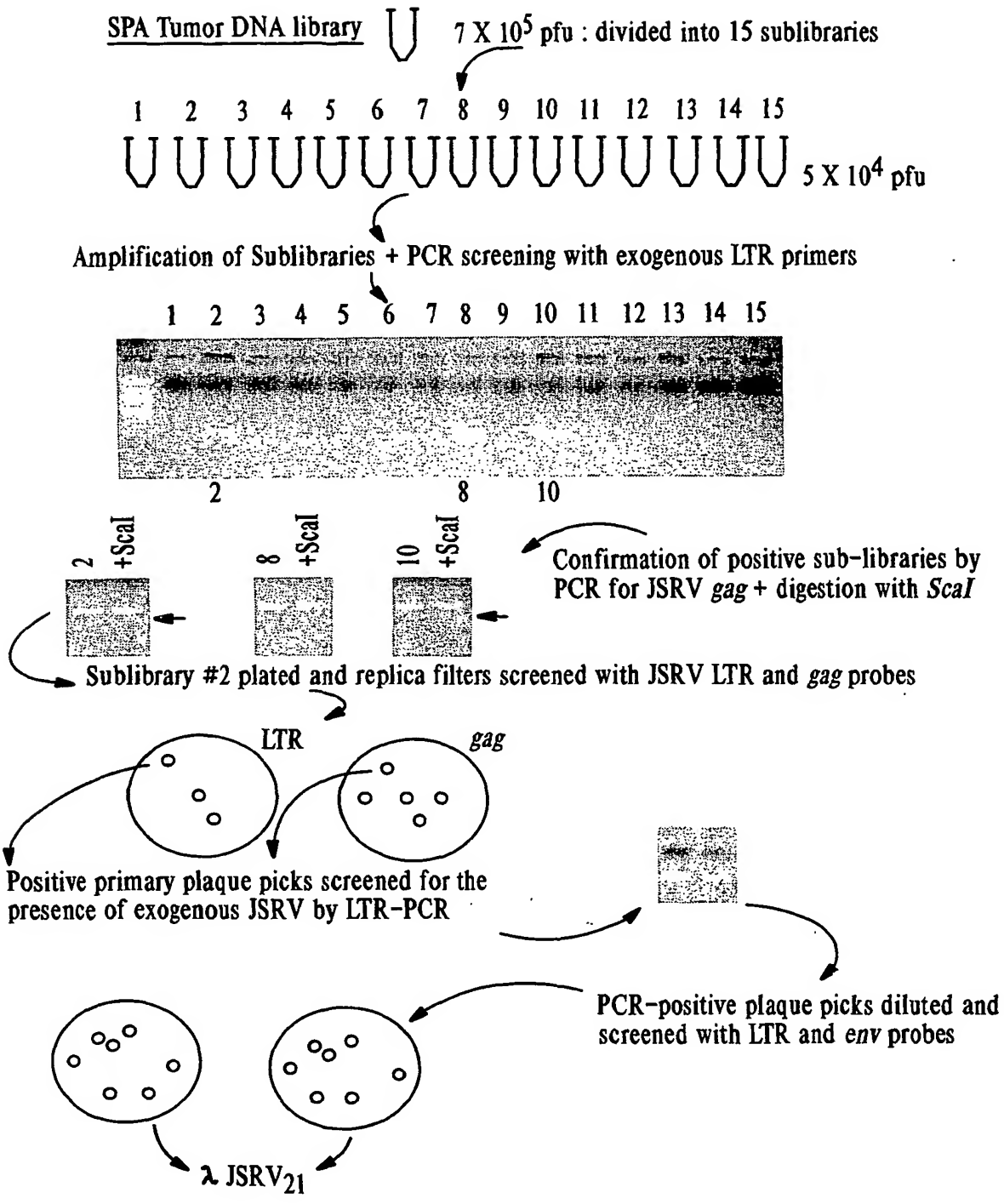

SPA Tumor DNA library
7 X 10^5 pfu : divided into 15 sublibraries
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
5 X 10^4 pfu
Amplification of Sublibraries + PCR screening with exogenous LTR primers
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
2
8
10
2 +ScaI
8 +ScaI
10 +ScaI
Confirmation of positive sub-libraries by PCR for JSRV gag + digestion with ScaI
Sublibrary #2 plated and replica filters screened with JSRV LTR and gag probes
LTR
gag
Positive primary plaque picks screened for the presence of exogenous JSRV by LTR-PCR
PCR-positive plaque picks diluted and screened with LTR and env probes
λ JSRV21

FIG. 1

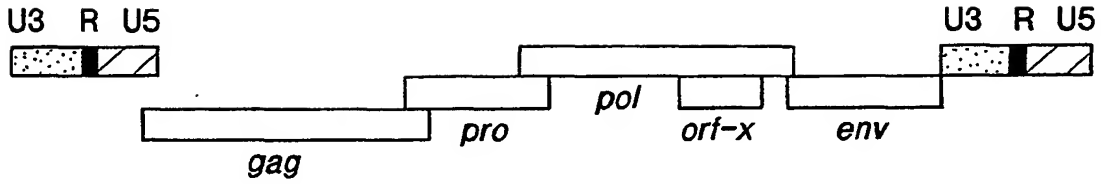

U3 R U5
U3 R U5
pol
pro
orf-x
env
gag

FIG. 2a

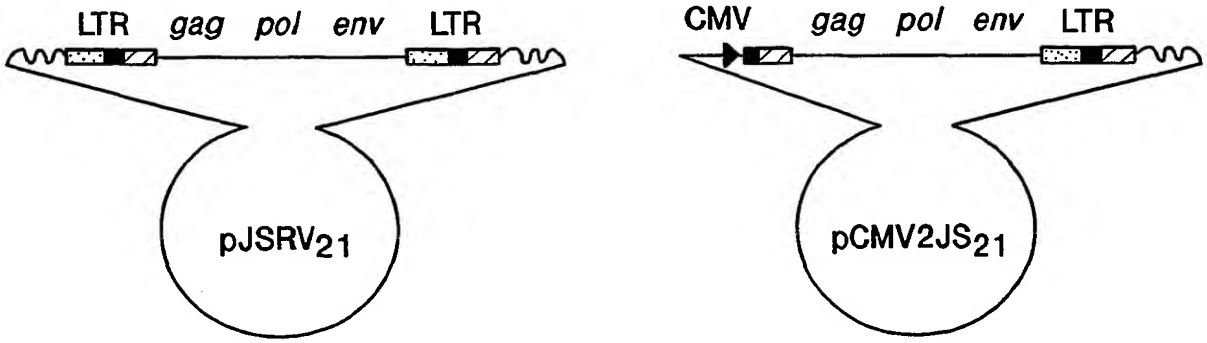

LTR gag pol env LTR
pJSRV21
CMV gag pol env LTR
pCMV2JS21

FIG. 2b

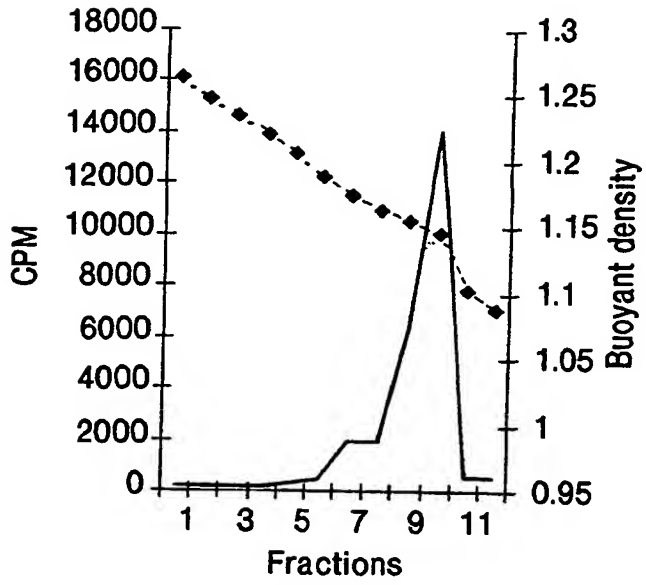

18000
16000
14000
12000
10000
8000
6000
4000
2000
0
CPM
1.3
1.25
1.2
1.15
1.1
1.05
1
0.95
Buoyant density
1 3 5 7 9 11
Fractions

FIG. 3a

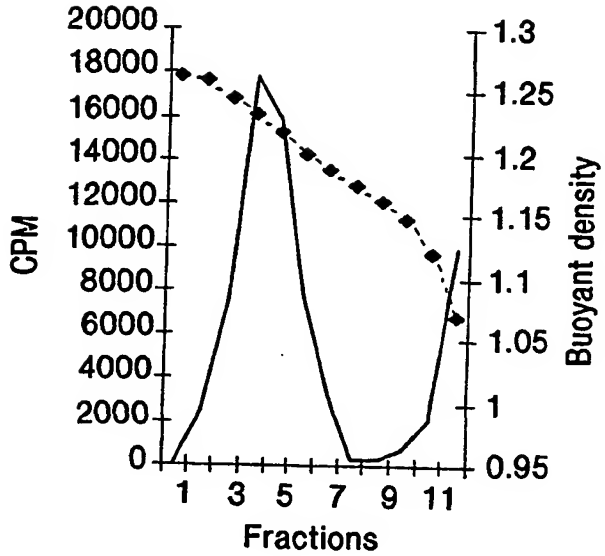

20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
CPM
1.3
1.25
1.2
1.15
1.1
1.05
1
0.95
Buoyant density
1 3 5 7 9 11
Fractions

FIG. 3b

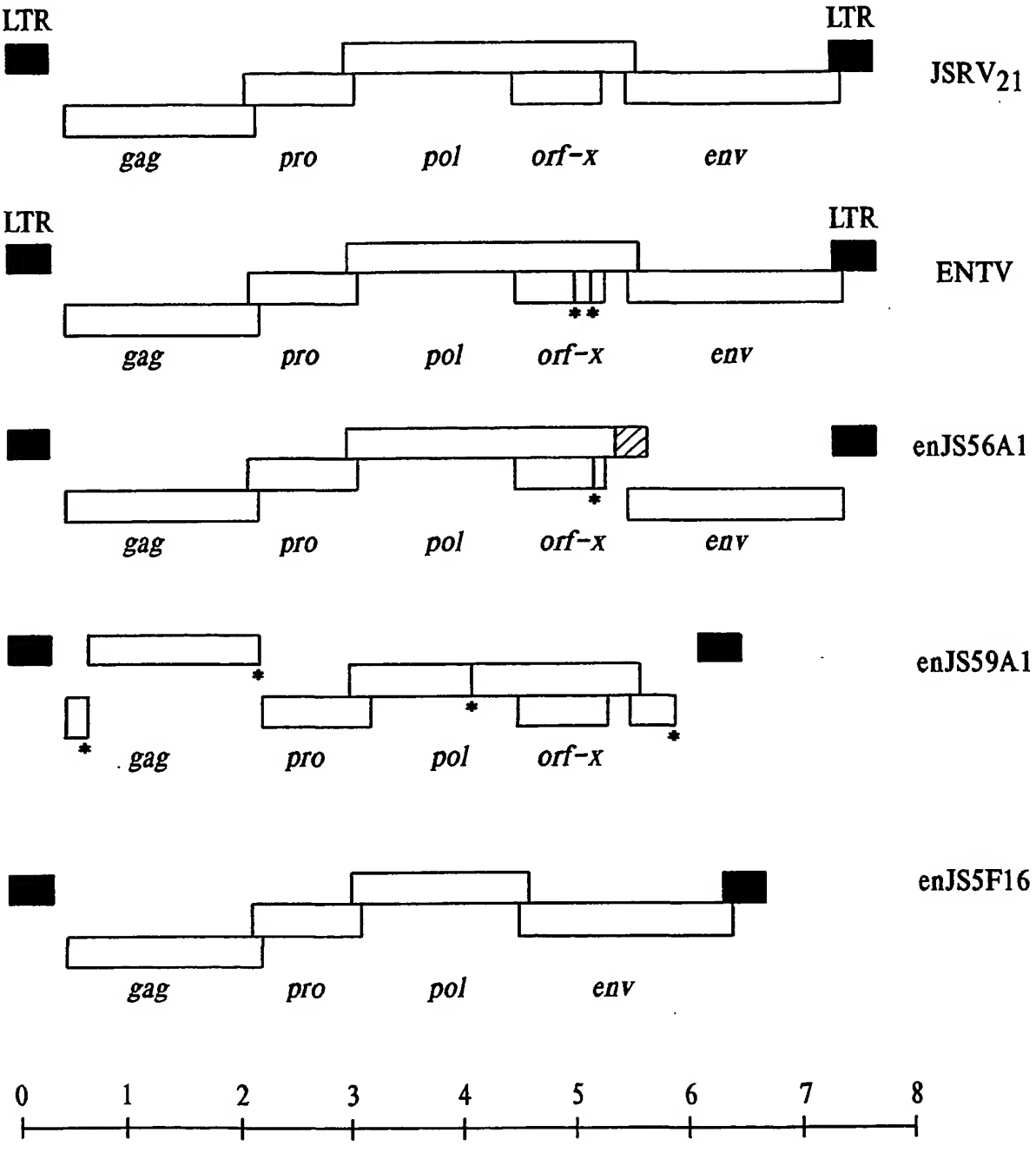

LTR
LTR
JSRV21
gag
pro
pol
orf-x
env
LTR
LTR
ENTV
gag
pro
pol
orf-x
env
enJS56A1
gag
pro
pol
orf-x
env
enJS59A1
gag
pro
pol
orf-x
enJS5F16
gag
pro
pol
env
0
1
2
3
4
5
6
7
8

FIG. 4

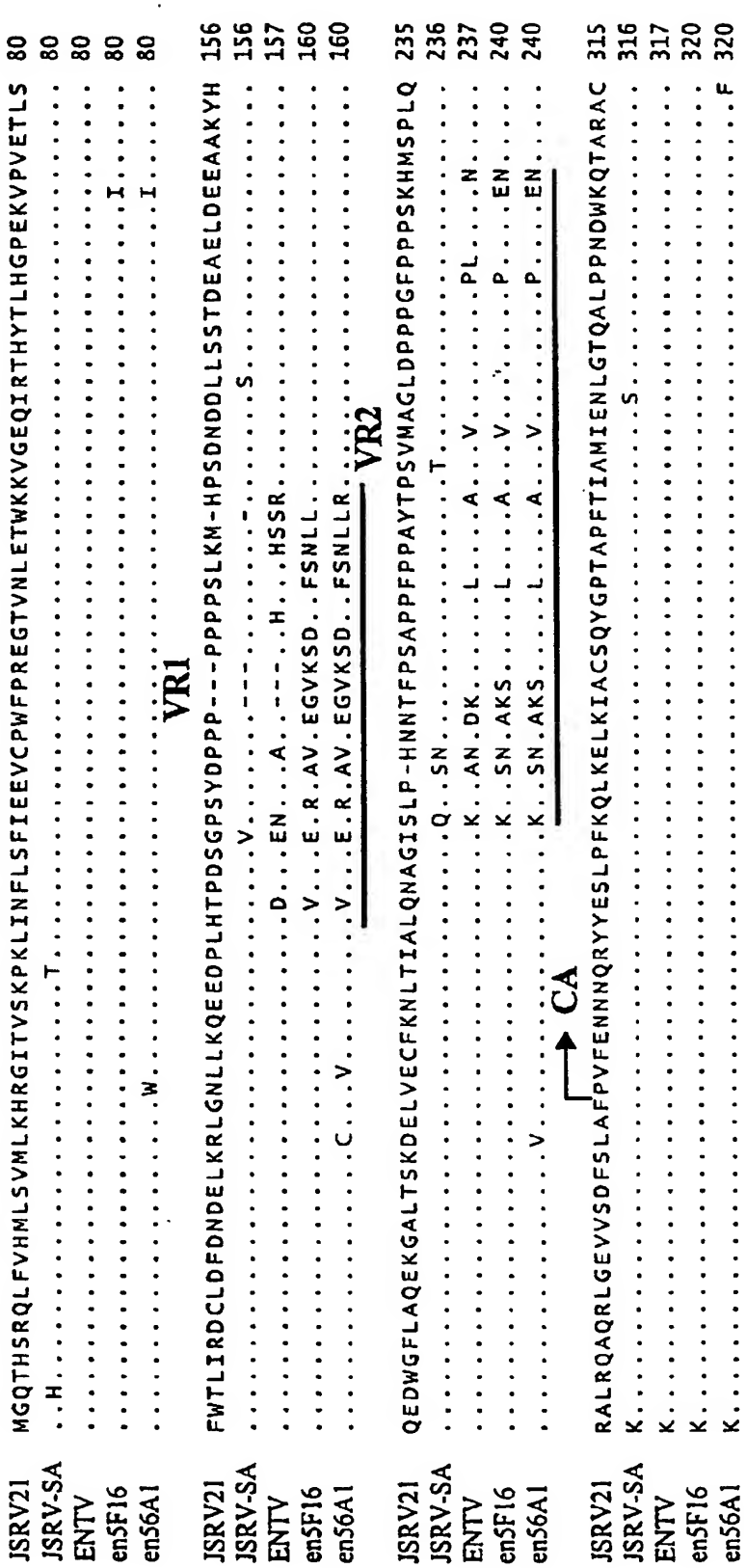

JSRV21 MGQTHSRQLFVHMLSVMLKHRGITVSKPKLINFLSFIEEVCPWFPREGTVNLETWKKVGEQIRTHYTLHGPEKVPVETLS 80
JSRV-SA ..H.....T..... 80
ENTV 80
en5F16 .....I..... 80
en56A1 .....W.....I..... 80
VR1
JSRV21 FWTLIRDCLDFDNDELKRLGNLLKQEEDPLHTPDSGPSYDPPP---PPPPSLKM-HPSDNDDLLSSTDEAELDEEAAKYH 156
JSRV-SA .....V.....---.....S..... 156
ENTV .....D...EN...A..---...H...HSSR..... 157
en5F16 .....V...E.R.AV.EGVKSD..FSNLL..... 160
en56A1 .....C...V.....V...E.R.AV.EGVKSD..FSNLLR..... 160
VR2
JSRV21 QEDWGFLAQEKGALTSKDELVECFKNLTIALQNAGISLP-HNNTFPSAPPFPPAYTPSVMAGLDPPPGFPPPSKHMSPLQ 235
JSRV-SA .....Q..SN.....T..... 236
ENTV .....K..AN.DK.....L.....A...V.....PL.....N..... 237
en5F16 .....K..SN.AKS.....L.....A...V.....P.....EN..... 240
en56A1 .....K..SN.AKS.....L.....A...V.....P.....EN..... 240
CA
JSRV21 RALRQAQRLGEVVSDFSLAFPVFENNNQRYYESLPFKQLKELKIACSQYGPTAPFTIAMIENLGTQALPPNDWKQTARAC 315
JSRV-SA K.....S..... 316
ENTV K..... 317
en5F16 K..... 320
en56A1 K.....F 320

FIG. 5-1

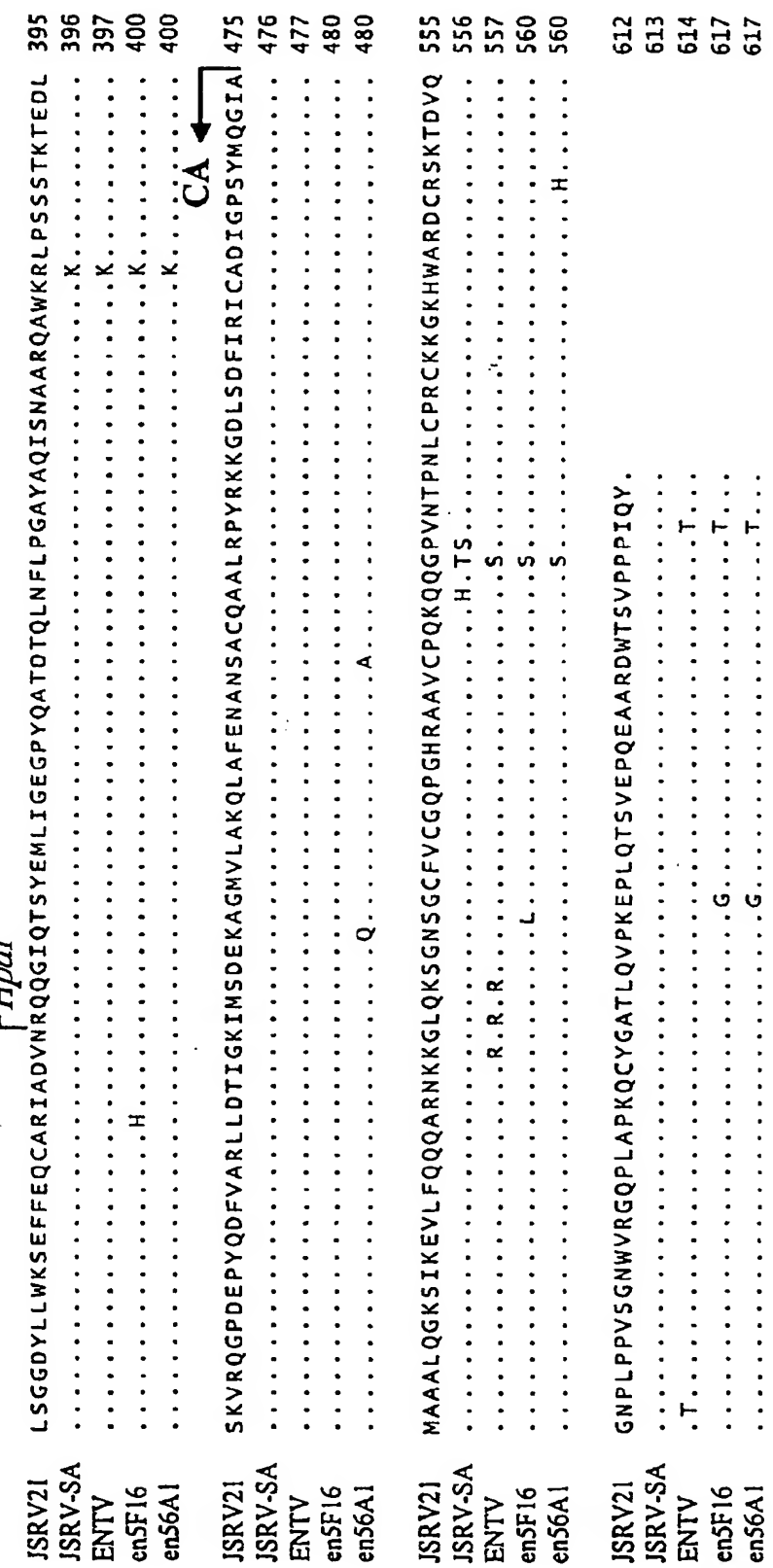

HpaI
CA
JSRV21 LSGGDYLLWKSEFFEQCARIADVNRQQGIQTSYEMLIGEGPYQATDTQLNFLPGAYAQISNAARQAWKRLPSSSTKTEDL 395
JSRV-SA ..... K..... 396
ENTV ..... K..... 397
en5F16 .....H..... K..... 400
en56A1 ..... K..... 400
JSRV21 SKVRQGPDEPYQDFVARLLDTIGKIMSDEKAGMVLAKQLAFENANSACQAALRPYRKKGDLSDFIRICADIGPSYMQGIA 475
JSRV-SA 476
ENTV 477
en5F16 480
en56A1 .....Q..... A..... 480
JSRV21 MAAALQGKSIKEVLFQQQARNKKGLQKSGNSGCFVCGQPGHRAAVCPQKQQGPVNTPNLCPRCKKGKHWARDCRSKTDVQ 555
JSRV-SA ..... H.TS..... 556
ENTV .....R.R.R..... S..... 557
en5F16 .....L..... S..... 560
en56A1 ..... S.....H..... 560
JSRV21 GNPLPPVSGNWVRGQPLAPKQCYGATLQVPKEPLQTSVEPQEAARDWTSVPPPIQY. 612
JSRV-SA 613
ENTV .T..... T... 614
en5F16 ..... G..... T... 617
en56A1 ..... G..... T... 617

FIG. 5-2

```
JSRV21    MPKRRAGFRKGWYARQRNSLTHQMQRMTLSEPTSELPTQRQIEALMRYAWNEAHVQPPVTPTNILIMLLLLLQRVQNGAA 87
JSRV-SA   ..........................................P..................................... 80
ENTV      ...H.............Y.......N.T.NG.....V.....H.........A..IK..........I..... 80
enJS56A1  ..................................................................I..... 87
enJS5F16  ..............HK..................................................I..... 87

JSRV21    AAFWAYIPDPPMIQSLGWDREIVPVYVNDTSLLGGKSDIHISPQQANISFYGLTTQYPMCFSYQSQHPHCIQVSADISYP 167
JSRV-SA   ..................................................................... 160
ENTV      ..................................................................... 160
enJS56A1  ..................K.T.............................................. 167
enJS5F16  ..................K.T.............................................. 167

JSRV21    RVTISGIDEKTGKKSYGNGSGPLDIPFCDKHLSIGIGIDTPWTLCRARVASVYNINNANATFLWDWAPGGTPDFPEYRGQ 247
JSRV-SA   ..................T..................................................... 240
ENTV      ............R..R..T.........N...S...........I.........T.L.................. 240
enJS56A1  ............R..RD.T.........................PI.........T.L.................. 247
enJS5F16  ............R..RD.T.........................I.........T.L.................. 247

JSRV21    HPPIFSVNTAPIYQTELWKLLAAFGHGNSLYLQPNISGSKYGDVGVTGFLYPRACVPYPFMLIQGHMEITLSLNIYHLNC 327
JSRV-SA   ....................................T..................................... 320
ENTV      ....L.......F..................................................... 320
enJS56A1  ..................................................................... 327
enJS5F16  ....L.......F..................................................... 327
```

FIG. 6-1

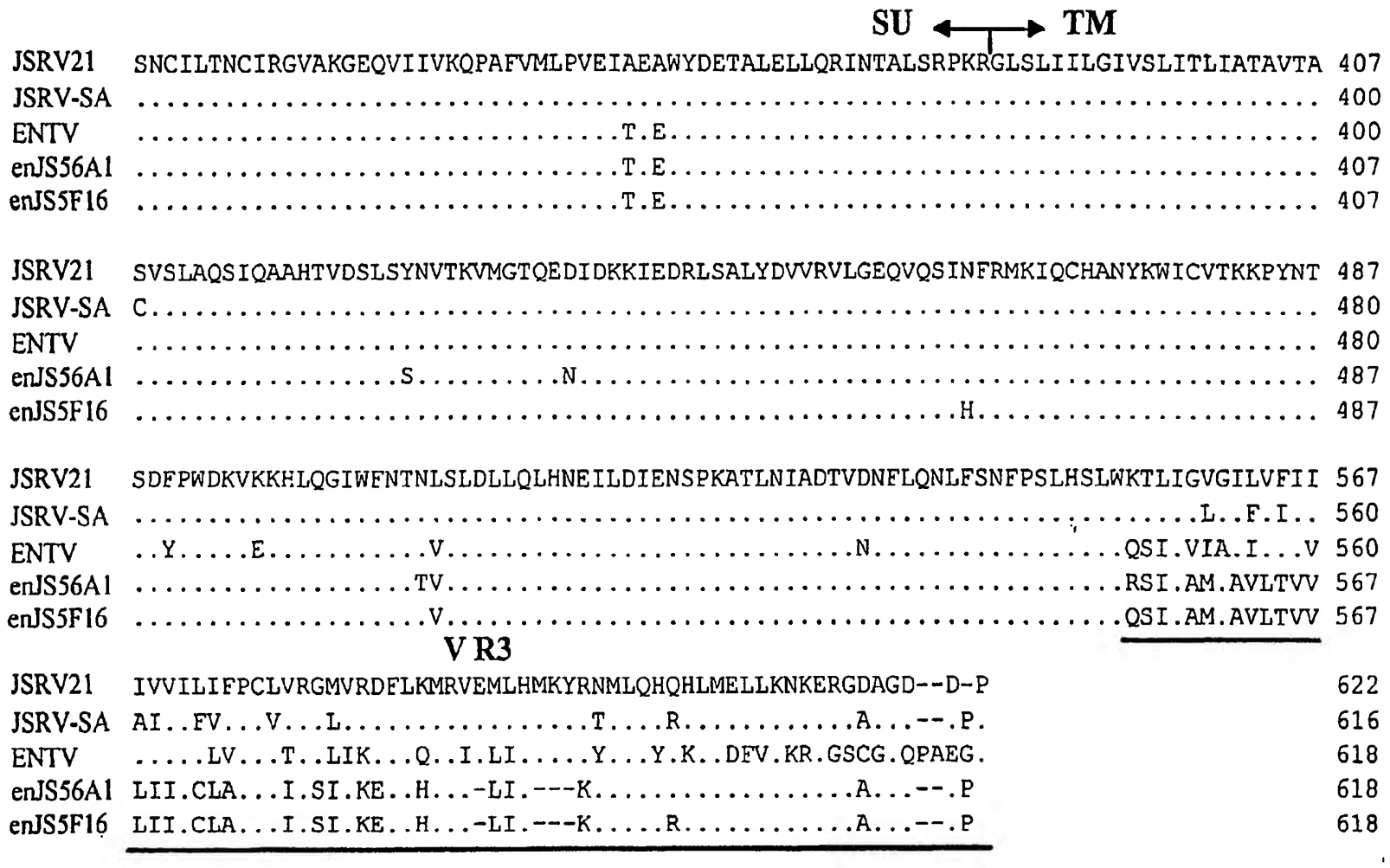


FIG. 6-2

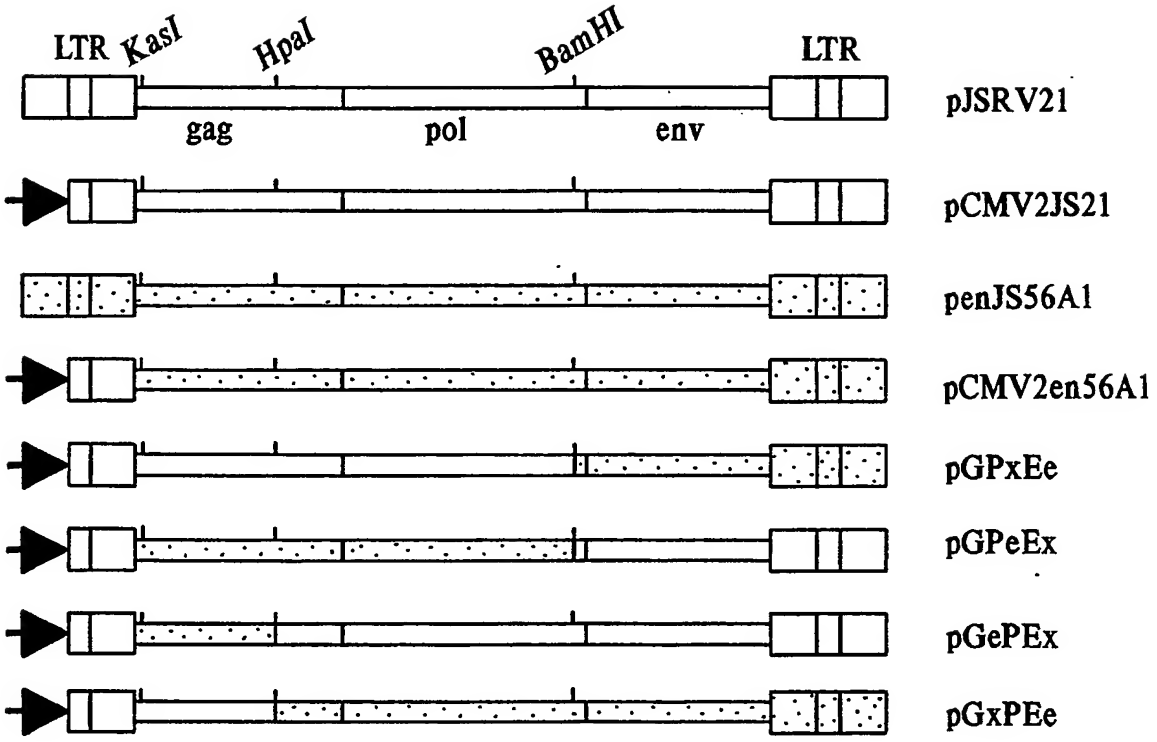

LTR
KasI
HpaI
BamHI
LTR
gag
pol
env
pJSRV21
pCMV2JS21
penJS56A1
pCMV2en56A1
pGPxEe
pGPeEx
pGePEx
pGxPEe

FIG. 7

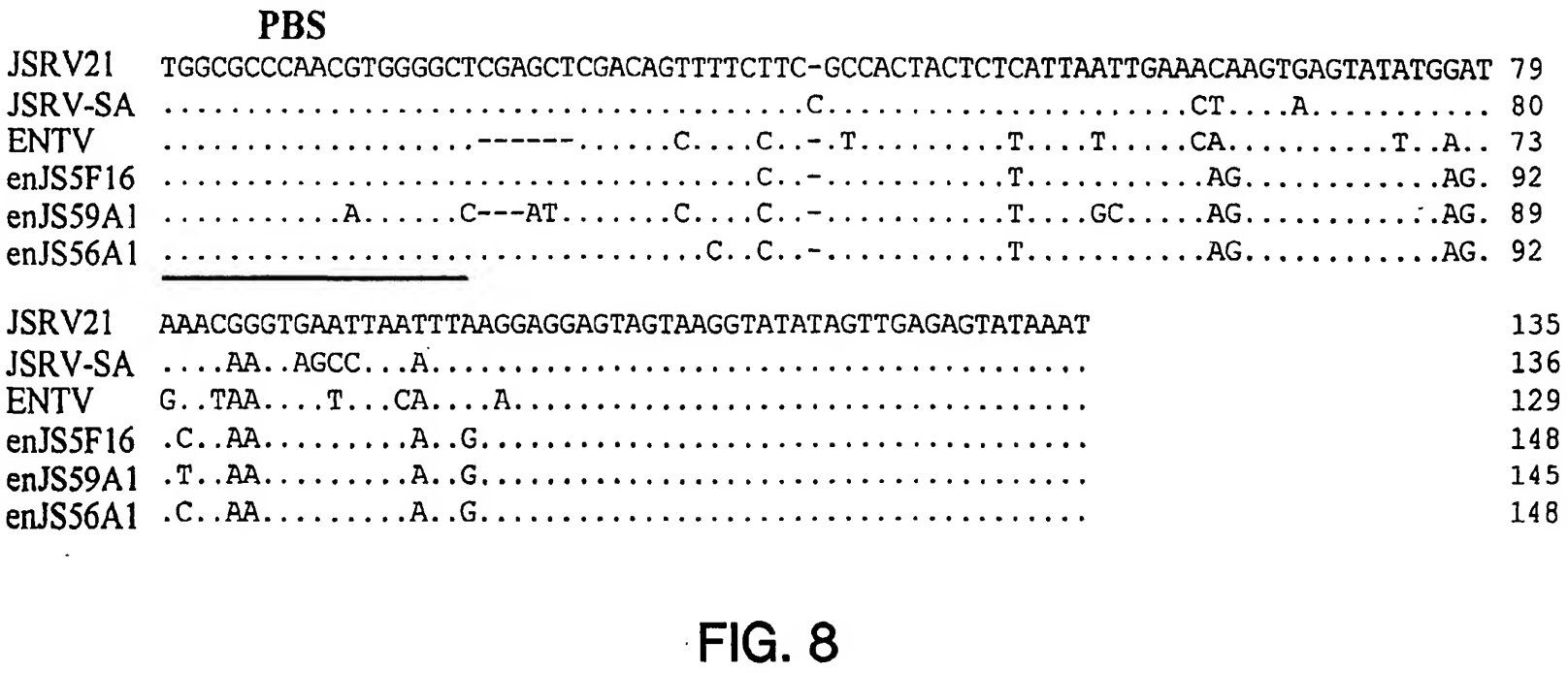

PBS
JSRV21 TGGCGCCCAACGTGGGGCTCGAGCTCGACAGTTTTCTTC-GCCACTACTCTCATTAATTGAAACAAGTGAGTATATGGAT 79
JSRV-SA .....C.....CT....A..... 80
ENTV .....------.....C....C..-.T.....T....T.....CA.....T..A.. 73
enJS5F16 .....C..-.....T.....AG.....AG. 92
enJS59A1 .....A.....C---AT.....C....C..-.....T....GC.....AG.....AG. 89
enJS56A1 .....C..C..-.....T.....AG.....AG. 92
JSRV21 AAACGGGTGAATTAATTTAAGGAGGAGTAGTAAGGTATATAGTTGAGAGTATAAAT 135
JSRV-SA ....AA..AGCC...A..... 136
ENTV G..TAA....T...CA....A..... 129
enJS5F16 .C..AA.....A..G..... 148
enJS59A1 .T..AA.....A..G..... 145
enJS56A1 .C..AA.....A..G..... 148

FIG. 8

FIG. 9A

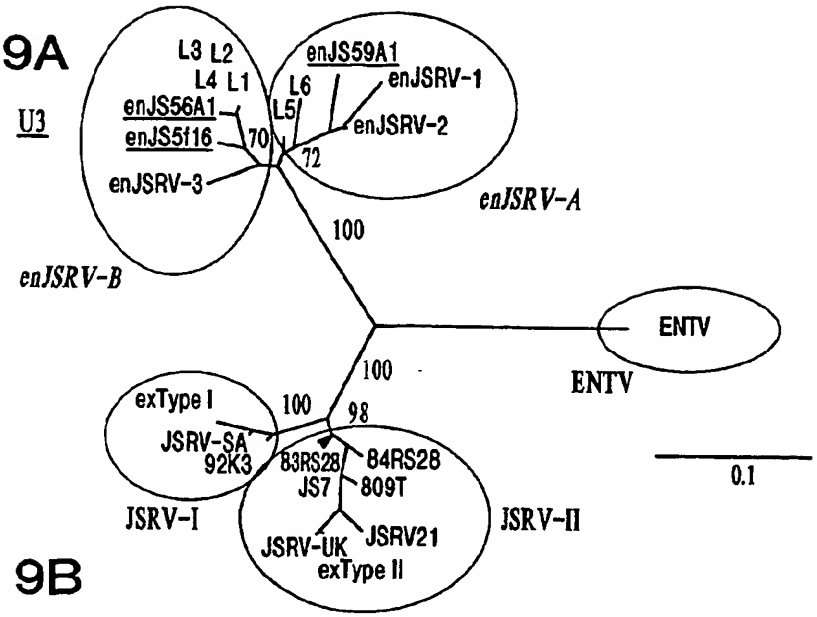


FIG. 9B

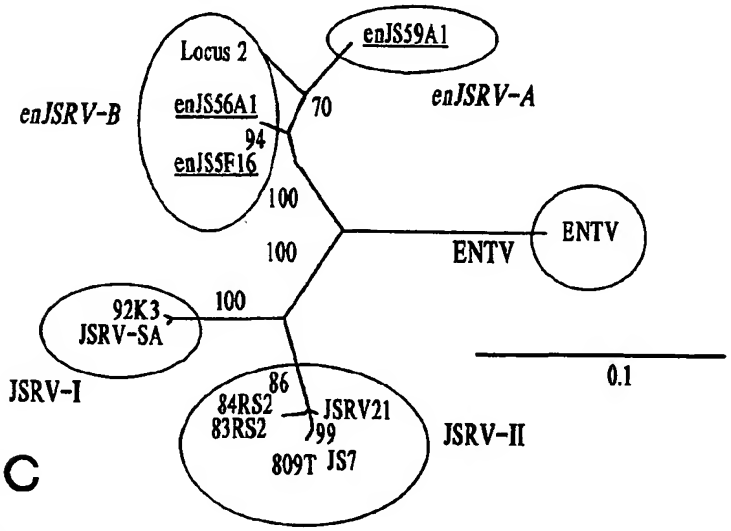
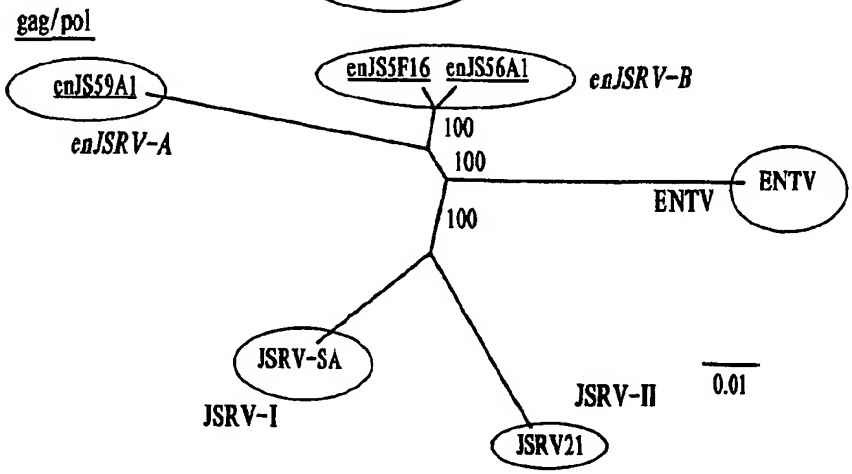


FIG. 9C



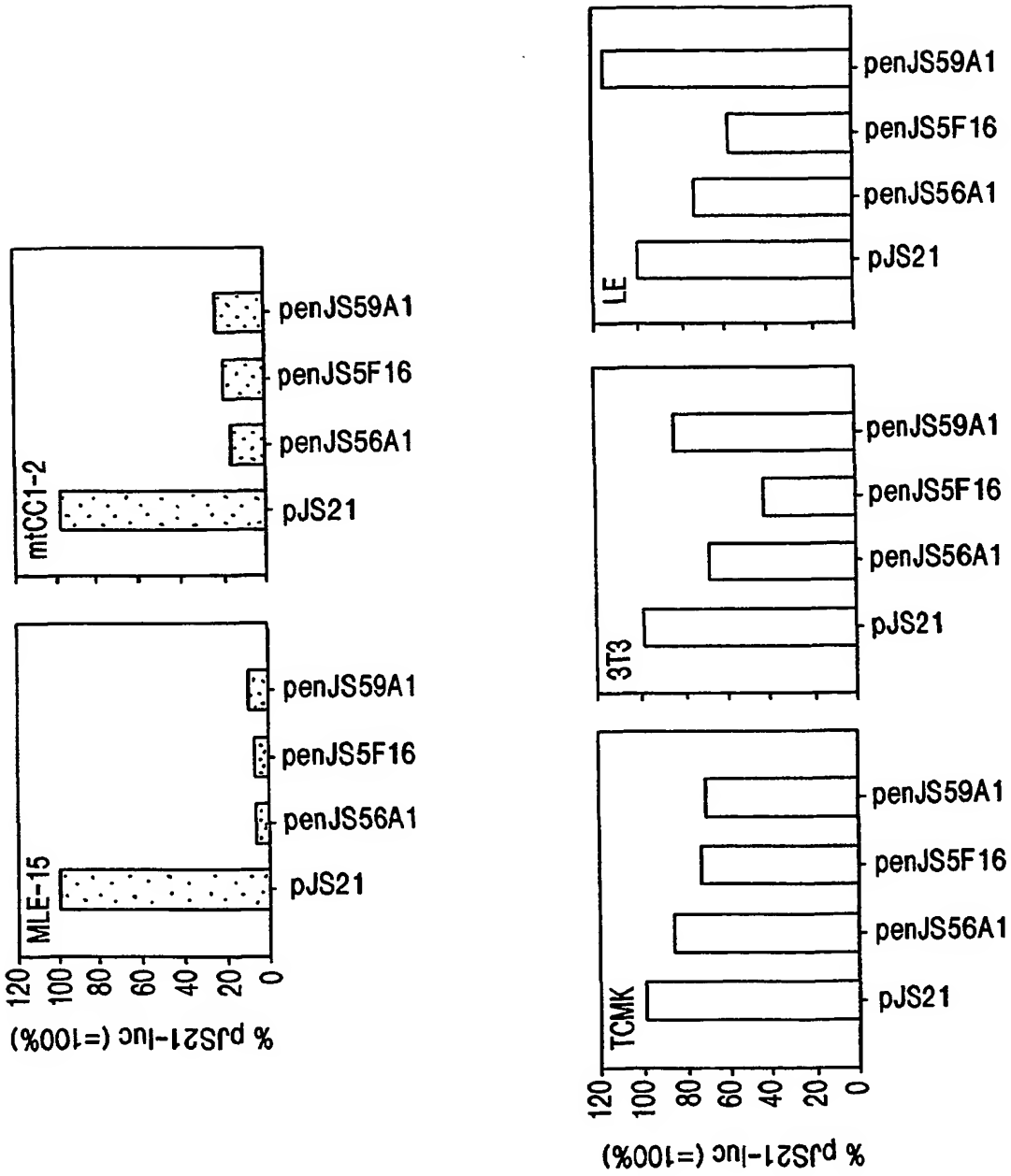

% pJS21-luc (=100%)
120
100
80
60
40
20
0
MLE-15
mtCC1-2
LE
3T3
TCMK
penJS59A1
penJS5F16
penJS56A1
pJS21

FIG. 10

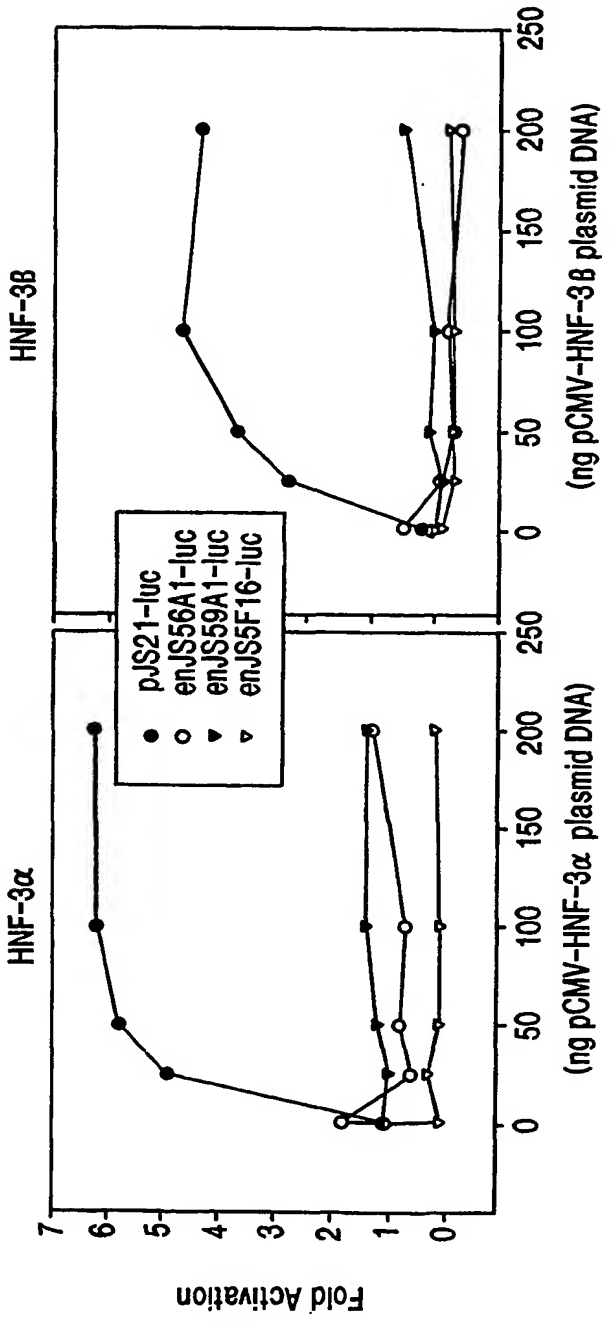

HNF-3α
HNF-3B
pJS21-luc
enJS56A1-luc
enJS59A1-luc
enJS5F16-luc
Fold Activation
0
1
2
3
4
5
6
7
0
50
100
150
200
250
(ng pCMV-HNF-3α plasmid DNA)
(ng pCMV-HNF-3B plasmid DNA)

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18856

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 1/12, 1/20
US CL : 435/235.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/235.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	YORK. D.F.et al. Isolation , identification, and partial cDNA cloning of genomic RNA of Jaagsiekte Retrovirus, the etiological agent of sheep pulmonary adenomatosis. J. Vir. September 1991. Vol. 65. No. 9. pages 5061-5067, especially abstract and pages 5061-5062.	1-3 --------- 3-24, 55-58
X ----- Y	YORK. D.F. et al. Nucleotide sequence of the Jaagskiekte Retrovirus, an exogenous and endogenous type D and B retrovirus of sheep and goats. J. Vir. August 1992. Vol. 66. No. 8. pages 4930-4939, especially abstract and pp. 4930-4931.	1-3 ---------- 4-24, 55-58
Y	US 5,849,718 A (GROSVELD) 15 December 1998, cols. 6-12.	4-16, 18-22, 24, 55-58

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 SEPTEMBER 2000	Date of mailing of the international search report 18 OCT 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRETT L. NELSON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18856

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,858,990 A (WALSH) 12 January 1999, cols. 15-16.	17
Y	STANDIFORD. T. J. et al Intermeukin-8 gene expression by a pulmonary epithelial cell line. J. Clin. Invest. December 1990. Vol. 86. pages 1945-1953, especially abstract.	23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18856

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, DIALOG, MEDLINE, BIOSIS, SCISEARCH, EMBASE
search terms: Jaagsiekte sheep retrovirus, JSRV, gag, pol, env, long terminal repeats, nucleic acid, vector, cell line, host, target, suicide, marker, cancer, thymidine kinase, plasmid, cmv early promoter

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups o
f inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-3, drawn to an isolated replication competent infectious Jaagsiekte sheep retrovirus.

Group II, claim(s) 4-12, drawn to a recombinant replication competent JSRV.

Group III, claim(s) 13-24 and 55-58, drawn to an isolated JSRV genome, an isolated polynucleotide, a vector and a method of producing an infectious JRSV.

Group IV, claim(s) 25-36, drawn to a method of treating a subject having a cell proliferative disorder.

Group V, claim(s) 37-42, drawn to a pharmaceutical composition comprising a JRSV polypeptide and method of inducing an immune response.

Group VI, claim(s) 43-49, drawn to an antibody an a method of inhibiting the binding of a JRSV to a cell employing the antibody.

Group VII, claim(s) 50-53, drawn to a method for identifying a compound which binds to JRSV.

Group VIII, claim(s) 54, drawn to a method of inhibiting the expression of JRSV.

Group IX, claim(s) 59, drawn to a method of driving lung-specific expression of a heterologous polynucleotide sequence.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I-III, V and VI recite different products which have different structures and activities and PCT rules 13.1 and 13.2 does not provide for multiple products.

Groups III-IX recite different methods which have different steps, employ different reagents and yield different results and PCT Rules 13.1 and 13.2 do not provide for multiple methods.